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Published quarterly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Entered as Second-Class Matter January 31, 1941, at the Post Office at Chicago, Illinois, under the Act of March 3, 1879. Subscription price: $4.00 per year for United States, Canada, and countries affiliated with the Pan American Postal Union; foreign countries, $4.30. Members of the American Veterinary Medical Association may subscribe to the American Journal of Veterinary Research for $2.50 per year added to the annual dues of $5.00. Single copies $1.25. Address all correspondence to American Veterinary Medical Association.

Vinylite-Resin-Corrosion Preparations in the Study of the Avian Leucosis Complex*

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A CLEAR CONCEPTION of the vascular system of a particular organ is very helpful in the study of lesions of that organ. Thus, methods for demonstrating the vascular system can be quite valuable adjuncts to the study of pathology. This is particularly true in studies on the pathogenesis of neoplastic diseases. In such cases, the distribution and finer intraorgan relationships of blood vessels and associated lesions cannot be readily visualized nor demonstrated by gross dissection or ordinary histological examination.

At least eight different methods for studying the vascular systems of organs have been described in the literature, namely: (1) gross dissections; (2) three dimensional reconstructions from serial histological sections; (3) transillumination of the living organ as developed by Knisely (1934, 1936, 1938) and MacKenzie *et al.* (1940, 1941); (4) x-ray examination of organs after injection of shadow-producing drugs as reported by Stiles (1897), Sampson (1912), and Edwards (1933); (5) the use of colored injection masses in organs rendered transparent by clearing as described by Shambaugh (1903) and Snapp (1924); (6) the use of infra-red photography in vascular studies of organs as discussed by Massopust (1934, 1936, 1937); (7) a combination of corrosion, histological and roentgenological methods using liquid rubber for the injecting medium (Lieb, 1940); and (8) the preparation of vascular casts.

Casts of the blood vessels were made as early as 1670 by Swammerdam and other contemporary workers (Narat *et al.*, 1936). Various substances have been employed to make these casts such as the different waxes, gelatin, plaster of Paris, Wood's metal, celloidin, celluloid, and more recently vinylite resin and liquid rubber. Narat *et al.* (1936) first reported the use of vinylite resin as a means of studying and demonstrating the vascular system. Puckett and Neumann (1940) further described the application of vinylite resin in the preparation of corrosion casts of anatomical specimens.

Scott and Moore (1928) pointed out the twofold use of injection methods in pathology: first, for purposes of research; and, second, for clear and concise demonstrations of vascular changes which would be difficult to demonstrate otherwise. The present study, using vinylite resin as the injection material, was initiated to investigate the vascular alterations in the organs of chickens affected with the avian-leucosis complex. The term, avian-leucosis complex, as adopted by a conference of research workers (1941), includes both leucosis (erythroblastic and granuloblastic), and lymphomatosis (neural, visceral, ocular, and osteopetrotic).

Leucosis is primarily a disease of the blood and blood forming organs (Feldman and Olson, 1933). The lesions of lymphomatosis, throughout their course, commonly show an intimate perivascular association (Johnson, 1934). This disease complex often produces considerable change in the size, shape, color and consistency of the organs affected. Thus, the possibilities for vascular alterations in this disease complex and the value of methods that demonstrate them, can be appreciated.

Only Single Comb White Leghorn Chickens were used in this study. A workable technique for demonstrating the vascular systems of normal organs was developed

*From the East Lansing Regional Poultry Research Laboratory, Bureau of Animal Industry, U. S. Department of Agriculture; presented before the Section on Poultry at the seventy-eighth annual meeting of the American Veterinary Medical Association, Indianapolis, Ind., August 11-15, 1941.

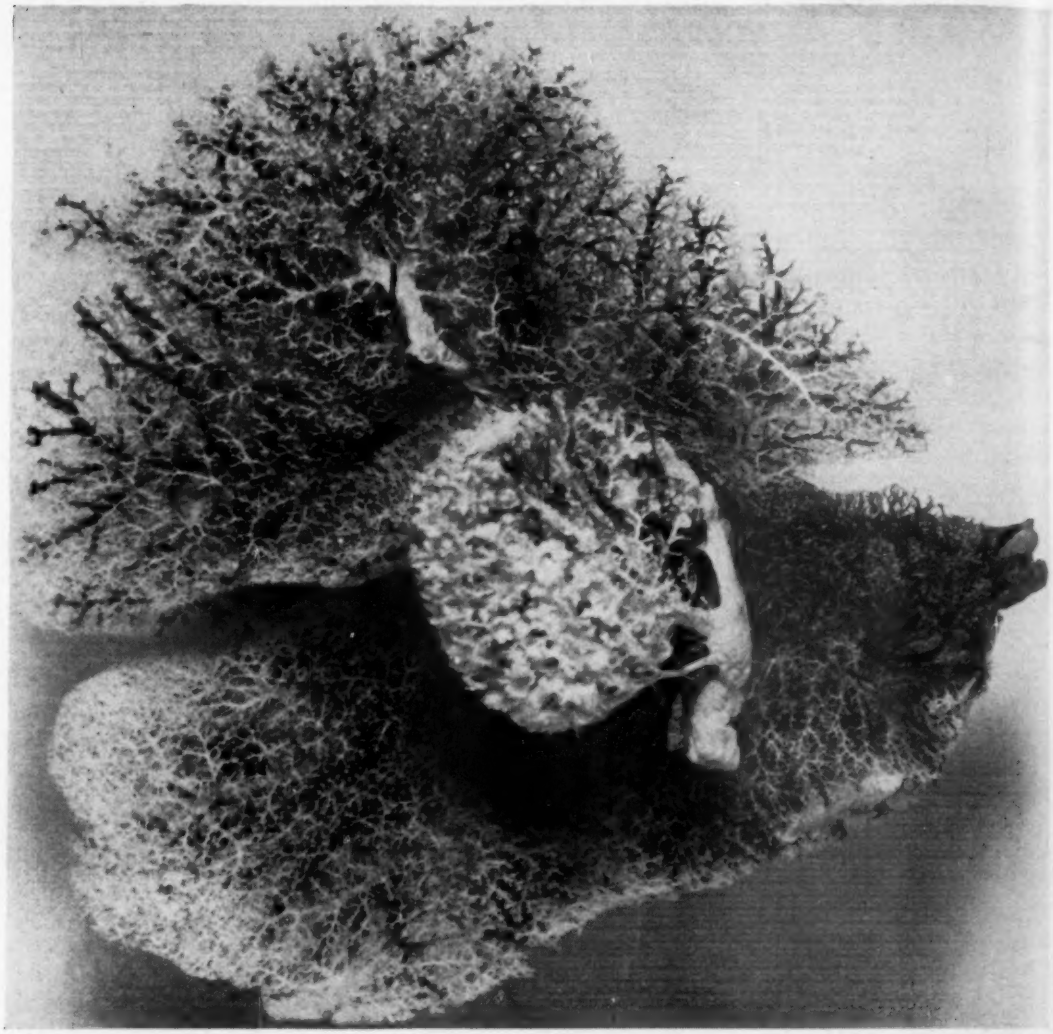

Fig. 1.—Dorso-posterior view of vascular cast of normal liver and spleen. The portal system (white), the hepatic vein (black), and the hepatic and splenic arteries (grey-black) are demonstrated.

first. Later the procedure was tried on pathological material.

TECHNIQUE

Vinylite resin, a plastic soluble in acetone, was purchased as a 12.5 per cent solution ready for use. Red, blue, green and clear solutions were utilized. The technique used for employing these solutions was similar to the method described by Puckett and Neumann (1940). The procedure that follows was found to be quite satisfactory and, in essential points, similar for most specimens. Therefore, in order to describe the technique, the normal liver of an adult domestic fowl was selected as the specimen, *par exemple*, to be injected. The production of vascular casts of other organs differed mainly in anatomical parts utilized.

The bird was killed by manual separation of the spinal cord and simultaneous rupture of the vessels of the cervical region. As soon as violent spasms ceased, the breast was removed to expose the viscera. The gizzard, liver, and attached structures were rotated to the right by severing their attachments to abdominal fat, peritoneum

and ligaments. The portal vein was cannulated as close to the liver as possible, using a glass cannula with a four-inch length of rubber tubing attached.

The aorta was cannulated posterior to the origin of the brachiocephalic arteries and a hemostat was applied to the aorta posterior to the ovary to eliminate blood vessels beyond this point from the injection. The adjacent veins were carefully avoided. The right atrium was then incised to form an exit for the blood and the subsequently injected water. The liver was rotated back to its original position. A 20-cc. glass syringe filled with the water was connected to the rubber tubing of the aortic cannula and the water slowly injected. The syringe was removed, refilled, connected to the cannulated portal vein and the water slowly injected. The process of alternating from aorta to portal vein was continued until the liver became pale grey and the water escaping from the incision in the right atrium was no longer blood tinged. Thus, most of the blood was flushed from the liver.

The foregoing procedures had to be performed quite rapidly in order to have the flushing well underway before large blood clots were formed in the vessels. To prevent an untimely precipitation of the resin that was soon to be injected, it was necessary to remove the water, because vinylite resin was precipitated from solution in

Fig. 2—Normal kidneys and adjacent vessels with skeletal parts retained to show relationships. The lighter colored vessels are veins and the dark vessels are arteries.

acetone when water was contacted. The water in the vessels was partially removed by injecting 15 cc. of acetone *via* each cannulated vessel. The excess acetone and water was forced out by injecting the air of several syringes into each vessel. The liver was then ready to receive the colored injection fluids.

A clean 20 cc. glass syringe was rinsed in acetone, filled with red vinylite resin solution, and the contents slowly injected into the cannulated aorta. Injection was discontinued when the red solution appeared in the small surface arteries throughout the liver. A hemostat was applied to the rubber tubing attached to the cannula to prevent escape of the fluid. The syringe was rinsed in acetone, filled with white vinylite resin solution, and the contents slowly injected into the portal vein until the small, surface, portal vessels were filled.

The posterior vena cava was then cannulated through the incision in the right atrium. As much of the residual acetone and water as possible was aspirated from the cannulated posterior vena cava and confluent hepatic vein with a glass syringe. Blue vinylite resin solution was then injected into the posterior vena cava, using the same technique as for the other vessels. The entire carcass was placed ventral side down in a pan of cold, tap water for 24 hours. By this time, the vinylite resin was solidified and had assumed the shape of the vessel lumen, thus forming a cast.

The cannulas were removed and all unnecessary parts were dissected from the specimen. The tissue was removed from the cast by corrosion in commercial hydrochloric acid for three days. A mat of glass wool was placed on top of the specimen to effect submersion. Unsightly stains difficult to remove from the casts resulted when specimens were only partially submerged. The cast was carefully removed from the acid with a ladle, gently washed under a stream of cold water, and placed on blotting paper to dry. The resultant multi-colored cast of the liver with red hepatic arteries, white portal veins, and blue hepatic veins was then ready for study under a low power microscope.

In the technique just described, vascular casts of the spleen and proventriculus also resulted, because it was deemed inadvisable to eliminate these two organs by using the smaller vessels closer to the liver for making the injections. Hence, the larger and more accessible vessels were chosen. As a final step the vascular casts of the proventriculus and spleen were sometimes separated from the liver cast by severing the casts of the portal vein radicles and the hepatic artery. However, leaving the casts intact afforded an opportunity to study the vascular relationships of the three organs. In the given method, the arterial systems of the gonads and adrenals were also injected; however, they were usually discarded.

The technique for injecting other organs differed mainly in anatomical parts utilized in making the injections. Vinylite resin, in the concentration used, did not penetrate the capillaries. For this reason it was possible to inject one system alone, *e.g.*, the hepatic venous system.

MODIFICATIONS OF THE TECHNIQUE

Other methods for inducing death were tried but in most cases the blood in the vessels seemed to clot more rapidly than in the technique previously described. The procedures tried were: chloroform inhalation, intravenous administration of 5 cc. of a 20 per cent sodium citrate solution, ether inhalation, and intravenous injection of halatal. Either of the former two agents was preferred when it was desired to leave the cervical vessels intact. Some satisfactory injections were made, using birds dead as long as 24 hours that had been chilled to a temperature of 5 to 8 C. However, better results were obtained when the animals were injected immediately after death.

Little success was attained in the injection of vinylite resin into the bile ducts or ureters because of their small size and the difficulty of removing the fluid content. The air passages of the lungs and the air sacs were injected *via* the trachea without much difficulty. Casts were also made of

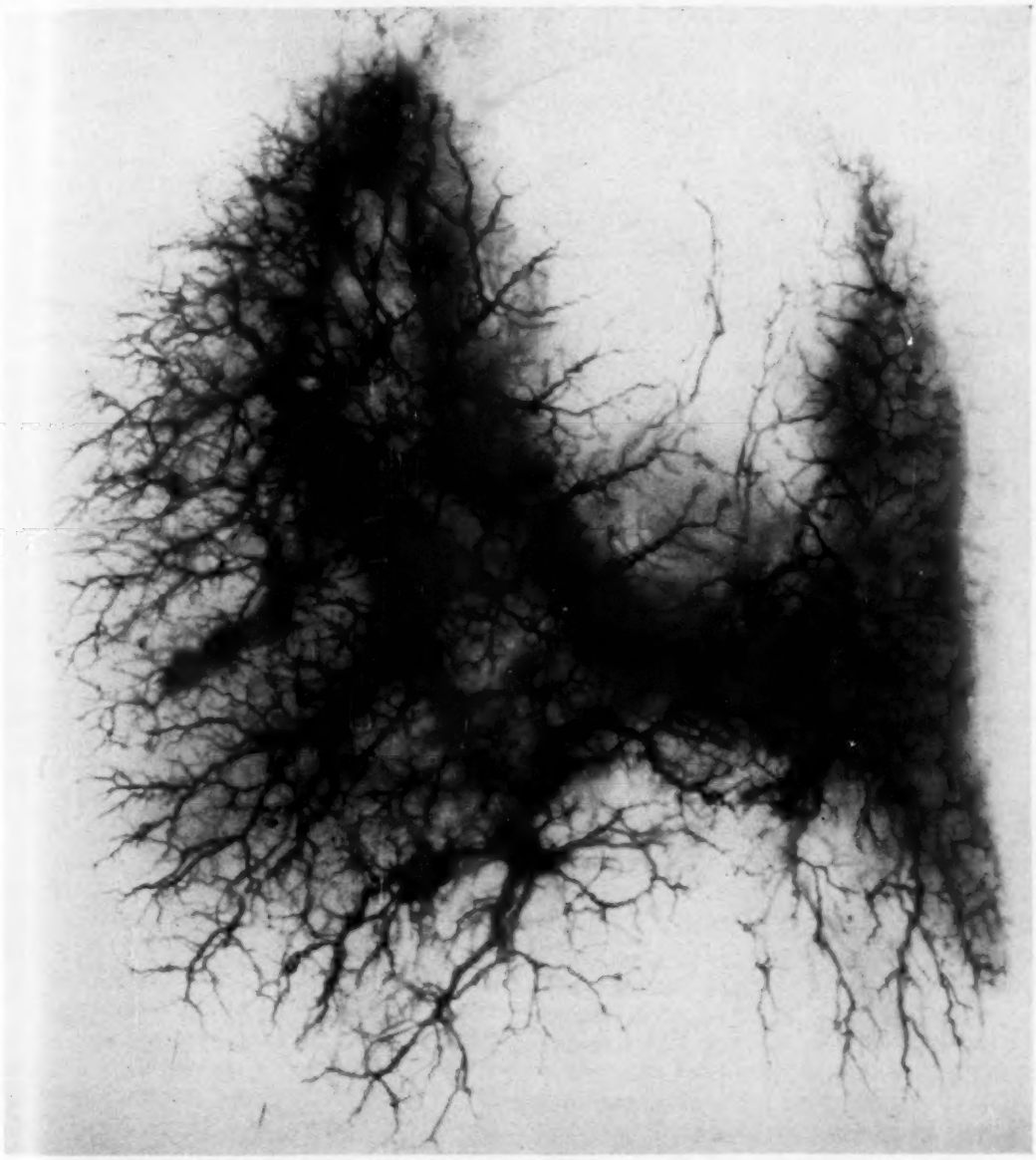

Fig. 3.—Cleared liver (portal vein injected) photographed by transillumination. The dark shadows on the right were probably produced by perivascular aggregations of tumor cells. (See text).

the chambers of the heart, the intestinal lumen, the sinuses of the head, and the secretory ducts of the uropygeal gland. A discussion of the technique for preparing the above specimens is beyond the scope of this paper.

In addition to the preparation of corrosion casts as described, two modifying techniques were employed. The first was a method to retain the skeletal parts adjacent to the vascular casts. It was found desirable to preserve the skeletal relationships of vascular casts of organs such as the kidneys and lungs. For such preparations, the technique as given was followed up to the point of placing in acid. Instead, the specimens, with muscles and other non-injected parts trimmed from the bones, were submerged in water in a covered crock. Bacterial action at room tempera-

ture for about one week loosened the soft tissues. The specimens were removed, gently washed under a stream of cold water, and placed in the sun to dry and bleach the bones. Specimens requiring additional bleaching were placed in commercial hydrogen peroxide for 24 hours and again dried in the sun. Ligaments were preserved to hold the bones together and before they dried it was necessary to orient the skeletal parts to a normal position.

The second modifying method was a procedure that allowed retention of the tissue in a transparent form. Thus, the vascular casts were visible within the cleared organs. This was the method of choice for intestinal preparations. The clearing technique was found to demonstrate quite well the intraorgan relationship of vessels and lesions in most organs.

An adaptation of the procedure employed in bone studies by Dawson (1926) was utilized. The injection procedure described was followed through the step of trimming away unnecessary parts. The specimens were then placed in 95 per cent alcohol for 48 hours. Clearing was started by submersion in a 1 per cent potassium hydroxide solution for 3 days. The specimens were next placed in Mall's solution (water 79 parts, glycerin 20 parts, and potassium hydroxide 1 part) for 1 day. Glycerin was added daily for the next 4 days to increase gradually the concentration of glycerine. After 4 days, clearing was complete and the specimens were transferred to a storage jar containing undiluted glycerin to which a crystal of thymol had been added. Permanent mounting in glycerin or removal for study was then possible.

DISCUSSION

It was noted that organs involved with the avian-leucosis complex were, as a group, easier to inject than normal organs, principally because of the increased size of the vessels and the ease with which the blood was removed.

While the blood was being flushed from livers, it was often observed that certain groups of sinusoids were immediately perfused. As a possible explanation, the theory that all capillary beds of an organ are not functioning simultaneously, that definite cycles of blood flow exist (Knisely, 1934), was considered. Thus, the first sinusoids or capillaries perfused may represent the groups that were last functioning during the terminal stages of death.

It was also noticed in the early stages of perfusion that a mottling of the organ was produced that closely approximated in color and shape certain liver lesions of lymphomatosis that are commonly observed. Livers with the blood replaced by water were grey. Likewise, the lesions of lymphomatosis, when devoid of blood, were almost the same shade of grey. Histological observations disclosed that capillary beds are quite sparse in the lesions of lymphomatosis. The casts, to be described later, disclosed that the lesions contained fewer vessels than the normal tissue. Thus, it may be concluded that the sharp color contrast between the lesions and the normal tissue is primarily produced by the difference in vascularity, and, therefore, blood content, of the two tissues. In other words, the neoplastic tissue probably would present a color similar to the normal tissue if it were vascularized to the same extent.

From the vinylite resin corrosion casts, it was noted that the arrangement of the major branches of veins and arteries was consistently similar for the normal chicken livers studied. Corrosion casts of the hepatic veins were made from the livers of two birds showing typical blood pictures of erythroblastic-granuloblastic leucosis. The livers were greatly enlarged, uniformly cherry red, and presented no grossly visible, focal lesions. The casts obtained from these livers showed an arrangement of major branches similar to the normal livers studied; however, in the former, the branches were much larger and farther apart.

A corrosion cast was prepared of the hepatic veins of a bird showing typical liver, kidney, and ovarian lesions of lymphomatosis. The diagnosis was verified from histological sections. A blood smear disclosed no abnormalities. The liver was greatly enlarged with very small indis-

Fig. 4.—A portion of an hepatic vein corrosion cast from a liver that had many large focal lymphomatosis tumors. A large tumor caused the compression and derangement of these vessels around the periphery of the tumor.

tinct, grey, focal areas uniformly distributed. The cast obtained from this liver was quite similar to the casts from the two birds with leucosis. The major branches of the hepatic vein, while similar in arrangement to the normal, were larger and farther apart.

In contrast with the above cases, livers showing large, distinct, focal lesions of lymphomatosis rendered vascular casts that were as erratic as the lesions. The lesion areas were observed to contain fewer blood vessels than the surrounding normal tissue. Corrosion casts demonstrated that the veins adjacent to the periphery of some of the larger lymphomatosis lesions were greatly flattened. Compression between the normal tissue and the growing neoplastic tissue, probably, was responsible for this abnormality.

When a liver involved with lymphomatosis was subjected to the clearing technique described, the normal tissue became transparent while the lymphomatosis lesions remained translucent. In the very mildly involved livers, small aggregations of translucent tissue were visible throughout the organ. The intimate association

with the veins and the general distribution of these translucent foci were thus demonstrated.

The technique described for retaining parts of the skeleton clearly demonstrated the blood supply of the bones and the relationship of adjacent organs. It seems probable that this method could be used to prepare a mounted specimen of a bird's skeleton which also demonstrates the entire circulatory system.

This preliminary report was primarily intended to present the procedure for preparing vinylite resin corrosion casts of the vascular system, and to point out the use of the procedure in a study of the avian-leucosis complex.

SUMMARY

The technique for using vinylite resin, to prepare corrosion casts of the vascular systems of organs of the domestic fowl, is described in detail. Two modifications of the technique are given: first, a method for retaining the skeletal parts adjacent to vascular casts; and second, a procedure for retaining the soft tissues surrounding the casts in a transparent form. These procedures were utilized to study the vascular alterations in chicken organs involved with the avian-leucosis complex. Hepatic vein corrosion casts of greatly enlarged livers, from two cases of leucosis and one case of lymphomatosis, were compared with casts from normal livers. A similarity in arrangement of major venous branches was noted; however, the branches were much larger and farther apart in the pathological cases. In cases showing large focal lesions of lymphomatosis, the casts obtained were very erratic, and the veins adjacent to the periphery of the larger lesions were observed to be flattened. The difference in vascularity is pointed out as the primary reason for the color contrast between the normal tissue and the lesions.

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Possible Irritating Effects from Dusting Baby Chicks and Growing and Adult Fowls with Hypochlorite Powders*

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MANY COMMENTS HAVE been made regarding the effects of dusting hypochlorite powders over baby chicks and adult birds affected with respiratory infections. It has been stated that pullorum-infected baby chicks whose lungs are involved are seriously affected when dusted with a hypochlorite powder. No basis for these statements could be found in the literature.

In view of the absence of scientific data, it was believed advisable to carry out extensive experiments covering various phases of the dusting of poultry with hypochlorite powders. Accordingly, a study was made of dusting:

a) Healthy baby chicks and growing birds.
b) Adult birds in production.
c) Chicks affected with infectious bronchitis (chick bronchitis).
d) Adult birds affected with bronchitis.
e) Adult birds affected with laryngotracheitis.
f) Adult birds affected with coryza.
g) Baby chicks affected with pullorum disease in which the lungs were involved.

Hypochlorite powders containing 15, 50, and 70 per cent available chlorine, sodium carbonate, and sodium chloride were dusted over poultry with an electric dust gun. The 15 and 50 per cent available chlorine products were prepared from 70 per cent calcium hypochlorite powder by diluting with either sodium carbonate or sodium chloride.

All powders were minus 200 mesh and, consequently, dusted easily, giving a heavy fog. Using these materials in dust form allows the determination of the causative factor or factors in the event irritation is produced.

*From the Research and Development Department, Pennsylvania Salt Manufacturing Company.

EXPERIMENTAL PROCEDURES

a) *Healthy Baby Chicks and Growing Birds.*—One hundred and fifty-four crossed Barred Rock Red day-old chicks were divided into 7 lots of 22 chicks each. They were leg banded and each group of 22 was weighed and placed in a coop 3′ x 3′ x 3′.

Seventy-five 6-week-old crossed Barred Rock Red chicks were divided into 7 lots of 7 birds each and 2 lots of 13 each. They were leg banded and each group weighed. Each group of 7 was placed in a coop 3′ x 3′ x 3′ and 13 in a coop 6′ x 3′ x 3′.

Each day for a period of 49 consecutive days the birds were dusted as shown in table I.

TABLE I

Lot 1	Hypochlorite powder	1) 50% available chlorine*
Lot 2	Hypochlorite powder	2) 50% available chlorine†
Lot 3	Hypochlorite powder	15% available chlorine†
Lot 4	Hypochlorite powder	70% available chlorine
Lot 5	Sodium carbonate	
Lot 6	Sodium chloride	
Lot 7	Control	

*Diluted with sodium carbonate.
†Diluted with sodium chloride.

The products were dusted into the coops with an electric dust gun (to rule out or minimize the human element) until a heavy fog was produced and the birds were sneezing and gasping. The coops were left closed for one hour and then opened to normal ventilation. Each week the mouths and upper respiratory tracts were examined for evidence of inflammation and each group of chicks weighed. On the forty-ninth day, postmortem examinations of several birds from each group were made to determine the presence or absence of inflammation in the respiratory passages.

b) *Adult Birds in Production.*—Eighteen Barred Rock hens in production were divided

into 3 lots of 6 birds each. Because of the danger of upsetting production by moving and changing quarters, the birds were kept for 2 weeks before egg records were tabulated. The production was recorded for 2 weeks and then dusting was started, as shown in table II.

TABLE II

Lot 1	Hypochlorite powder	1) 50% available chlorine
Lot 2	Hypochlorite powder	2) 50% available chlorine
Lot 3	Control	

The birds were dusted for 21 successive days with the products, the electric duster being used. The mouths and upper respiratory tracts were examined frequently. Production records were kept.

c) *Chicks Affected with Infectious Bronchitis (Chick Bronchitis).*—Two groups of 40 New Hampshire Red chicks, 4 days old, were infected with infectious bronchitis by swabbing the nasal passages and upper tracheas with infectious material from known bronchitis-affected chicks. Seven days later, gasping and sneezing were general in both groups. The two groups were divided into four lots of ten chicks each and dusted as recorded in table III.

TABLE III

Lot 1	Hypochlorite powder	1) 50% available chlorine
Lot 2	Hypochlorite powder	2) 50% available chlorine
Lot 3	Hypochlorite powder	15% available chlorine
Lot 4	Control	

The chicks were dusted (electric duster) with the products for seven successive days. The respiratory tracts of all chicks which died during the experiment and several from each group as soon as dusting was discontinued on the seventh day were examined for lesions.

d) *Growing Birds Affected with Infectious Bronchitis.*—Forty-three Barred Rock birds, 3 months old, were infected with bronchitis virus by swabbing the nasal passages and tracheas with a masseurated bronchitis tissue culture. Eight days later, watery eyes, gasping and sneezing were general throughout. They were divided into lots and dusted as shown in table IV.

TABLE IV

Lot 1	Hypochlorite powder	1) 50% available chlorine (13 birds)
Lot 2	Hypochlorite powder	2) 50% available chlorine (7 birds)
Lot 3	Hypochlorite powder	15% available chlorine (7 birds)
Lot 4	Control	

After being dusted for 4 successive days, the birds in all four lots were improved. Several from each group were immediately killed and examined.

e) *Young Birds Affected with Laryngotracheitis.*—One hundred and eight chickens, 2½ to 3 months old, were infected with laryngotracheitis by brushing the nasal passages and tracheas with the virus of laryngotracheitis. They were divided into groups and treated as follows:

Lot 1: Twenty birds were dusted immediately with a hypochlorite powder containing 50 per cent available chlorine to determine if such a procedure would increase the severity of the infection. The dusting procedure was continued for 12 successive days.

Lot 2: Forty birds were dusted with a hypochlorite powder containing 50 per cent available chlorine as soon as the infection appeared (usually 48 hours after exposure). Dusting was continued for 9 successive days.

Lot 3: Forty-eight birds—control.

Immediately after dusting was discontinued, several from each of the 3 lots were killed and examined.

f) *Young Birds Affected with Coryza.*—Thirty-six birds, 3 to 4 months old, were infected with coryza by brushing the nasal passages and tracheas with the coccobacillary coryza agent. The birds were divided into groups and treated as follows:

Lot 1: Nine birds were dusted immediately with a hypochlorite powder containing 50 per cent available chlorine and then dusted daily for 20 successive days.

Lot 2: Thirteen were dusted daily for 8 successive days after the infection appeared, which was 10 days after inoculation.

Lot 3: Fourteen birds—control.

Immediately after dusting was discontinued, several from each group were killed and examined.

g) *Baby Chicks Affected with Pullorum Disease Involving the Lungs.*—One hundred and fifty-two baby chicks from pullorum-clean flocks were infected with *Shigella gallinarum* by atomizing a 24-hour broth culture over them.

CHART I—RATE OF GROWTH OF CHICKS

1. Hypochlorite powder No. 1—50% Avail. chlorine.
2. Hypochlorite powder No. 2—50% Avail. chlorine.
3. Hypochlorite powder —15% Avail. chlorine.
4. Hypochlorite powder —70% Avail. chlorine.
5. Sodium chloride
6. Sodium carbonate
7. Control

Each shaded area represents week's growth.

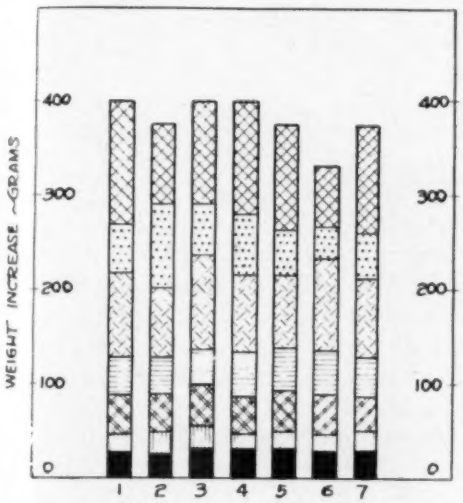
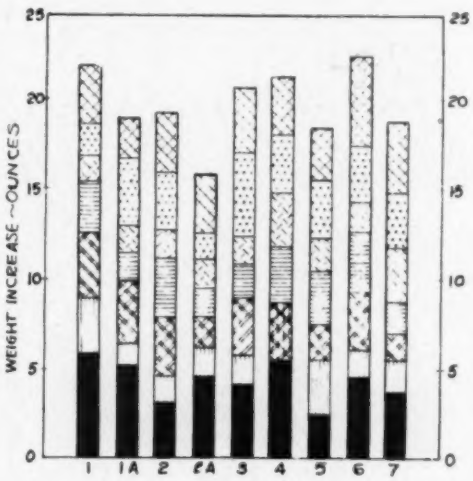


CHART II—RATE OF GROWTH OF GROWING BIRDS

1. Hypochlorite powder No. 1—50% Avail. chlorine.
2. Hypochlorite powder No. 2—50% Avail. chlorine.
3. Hypochlorite powder —15% Avail. chlorine.
4. Hypochlorite powder —70% Avail. chlorine.
5. Sodium chloride
6. Sodium carbonate
7. Control



They were divided into 2 lots as soon as the infection appeared and treated as follows:

Lot 1: Seventy-six of them were dusted daily for 13 successive days with a hypochlorite powder containing 50 per cent available chlorine.

Lot 2: Seventy-six chicks—control.

Immediately after dusting was discontinued, several from each group were killed and examined. Postmortem examinations also were made on all chicks which died during the experiment.

DISCUSSION

a) At no time during the dusting or on postmortem examination did the healthy chicks or growing birds show evidence of inflammation in the respiratory tract. Charts 1 and 2 give the percentage gains in weight. It can be seen that the dusted birds gained as well as the controls, indicating that dusting with 15, 50, or 70 per cent available chlorine hypochlorite powders, sodium carbonate, or sodium chloride did not affect the growth rate.

b) Examinations of the respiratory tracts of the laying birds showed no evidence of inflammation. Chart 3 gives the production record, which shows very little difference in production within the groups.

c) Chicks affected with infectious bronchitis and dusted with the hypochlorite powders showed less severe lesions than the controls. More severe lesions and more mucus were found in the upper respiratory tracts of the birds dusted with the 15 per cent powder than those dusted with the 50 per cent products, but less than those found in the controls. The mortality in the control group was 20 per cent as compared with 10 per cent in the dusted group. If the powders had caused more inflammation in the respiratory tract, more severe lesions would have been found in the dusted chicks, probably with a higher mortality.

d) The growing birds affected with bronchitis showed less severe lesions in the dusted group than in the control group. More severe lesions and more mucus were present in the upper respiratory tracts of the birds dusted with the 15 per cent powder than those dusted with the 50 per cent products, but less than in the controls. In

TABLE V—Mortality During the 13-Day Dusting Period

Day	Lot 1		Lot 2		Lot 3		Lot 4	
	Dusted	Control	Dusted	Control	Dusted	Control	Dusted	Control
1							0	0
2							0	0
3							0	0
4							0	0
5					1		0	0
6				4	1	1	0	0
7		1	2		5	2	0	0
8					2		0	0
9					2	2	0	0
10			1		2	2	0	0
11							0	0
12			1	1	2	6	0	0
13				1		2	0	0

the control group of 13 birds, one died of bronchitis. There were no deaths in the dusted groups of 20 birds.

e) In the adult birds, difficulty was experienced in producing a laryngotracheitis infection severe enough to cause death. However, severe gasping and sneezing were produced. Here again in this group, the dusted birds did not have more severe lesions than the controls, and in most instances they were less severe. The birds which were dusted heavily from the day of inoculation did not develop more severe infection than those dusted after the infection appeared.

f) The dusting of adult birds affected with coryza did not produce more severe lesions than those in the controls. The infection developed slowly and the birds dusted heavily from the day of inoculation did not show more severe inflammation than those dusted after the infection appeared.

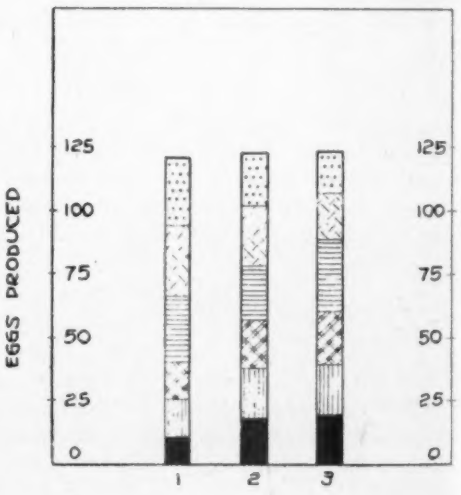
g) Lung lesions caused by *Sh. gallinarum* could be produced by atomizing a 24-hour broth culture over the chicks. A more severe infection was produced in the chicks 4 to 6 days old than in the day-old chicks. Dusting the chicks for 13 successive days after symptoms were noted did not cause further injury (table V). The lesions in the control birds were more severe than those in the dusted group.

In this study, the dusting of hypochlorite powder containing 15, 50, and 70 per cent available chlorine was continued for much longer periods of time than under practical conditions. Frequent examinations of the healthy and diseased birds during the test periods showed that inflammation was not produced by the dustings.

CHART III—EGG PRODUCTION RECORD

1. Hypochlorite powder No. 1—50% Avail. chlorine.
2. Hypochlorite powder No. 2—50% Avail. chlorine.
3. Control

Each shaded area represents week's growth. Shaded areas represent week's production of eggs. No dusting during 1st, 2nd and 6th weeks.



SUMMARY

Studies were made of the possible irritating effects of dusting hypochlorite powders heavily on several successive days over healthy chicks and adult birds as well as birds affected with respiratory diseases.

Examinations of the healthy dusted and control birds revealed no evidence of respiratory inflammation. The growth and production in these groups were the same as in the controls.

Dusting chicks affected with infectious bronchitis or older birds with bronchitis, laryngotracheitis, or coryza with hypochlorite powders did not cause more severe inflammation than that produced by the infective agents.

Pullorum-infected chicks whose lungs were involved were not adversely affected by the dusting, as shown by the higher mortality in the control birds.

Respiratory-Nervous Disorder in Eight-Months Old Pullets

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Petaluma, California

THREE WHITE LEGHORN pullets, 8 months old, were brought to this laboratory in April, 1942. The owner of the flock reported that during the three days previous to his visit to the laboratory approximately 70 per cent of the flock of 750 birds became sick. The symptoms were blueness of the comb, droopiness, inappetence and a tendency for many of the birds to remain on the roosts. Egg production dropped from 64 to 17 per cent, and a number of soft-shelled eggs were laid. When questioned, the owner indicated that there was a lack of respiratory or nervous symptoms. Six birds died, presumably as a result of the disease.

The three pullets presented for examination had a marked cyanosis of the combs and were otherwise visibly ill. Because of the occurrence in this region of numerous outbreaks of an infectious respiratory-nervous disorder of chickens of all ages,[1] particular attention was paid to the voices, respiration and muscular movements of the specimens examined. There were no functional disturbances that could be detected. Autopsies of the birds disclosed, however, that the air sac membranes and the mesentery in all three specimens were cloudy in appearance and slightly edematous. A considerable amount of straw together with particles of the regular feed was found in the crops. No other lesions were detected.

Inasmuch as the condition of the mesentery and of the air sac membranes in the affected pullets was similar to that seen in many cases of the respiratory-nervous disorder, an attempt was made to determine if the disease in the pullets was caused by the same filtrable virus. Lung and air sac tissues from the three specimens were pooled, macerated and then mixed with approximately 6 cc. of physiologic salt solution and filtered through gauze. Each of three chicks, ten days old, was inoculated intranasally with a few drops of this material. Within six days after inoculation two of the chicks showed respiratory and nervous symptoms typical of those associated with the respiratory-nervous disorder. One of the affected chicks was destroyed and was found to have infected air sacs and a congestion and edema of the lungs. The other affected chick recovered in approximately three weeks after the appearance of the first symptoms. The recovered chick and the one which apparently had not contracted the disease were then inoculated with a known strain of the virus of the respiratory-nervous disorder and proved to be refractory. Three other chicks from the same source and of the same age as the

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[1]Stover, D. E.: A filterable virus, the cause of a respiratory-nervous disorder of chickens. Amer. J. Vet. Res., iii (1942), pp. 207-213.

inoculated chicks served as uninoculated controls during the transmission experiment and when inoculated later all were found to be susceptible to the respiratory-nervous disorder.

This case illustrates an atypical type of respiratory-nervous disorder without respiratory or nervous sypmtoms. The clinical manifestations correspond to those

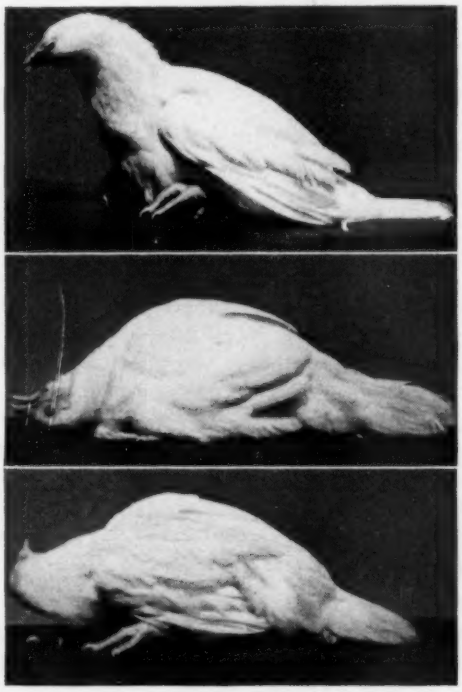

Fig. 1—Three pictures showing an artificially infected White Leghorn pullet. From above downward, the tortocollis changed rapidly from the 1st, 2nd, and 3rd positions shown.

described in the eastern portion of the United States as "blue comb" or "pullet disease." Jungherr and Levine[2] made an extensive investigation of the pathology of so-called pullet disease and were the first to call attention to this syndrome. Brunett and Bullis,[3] Beaudette,[4] and Stafseth[4] subsequently reported additional outbreaks. In southern California, the Los Angeles County Live Stock Department[5] reported an outbreak of disease in White Leghorn pullets which in its clinical aspects resembled so-called pullet disease. In the same section of California, Jones and Harr[6] studied a number of outbreaks of disease in chickens in which the most characteristic symptom displayed was cyanosis of the comb. The cause of these outbreaks of disease in the eastern portion of the United States and in southern California has not been established.

The fact that the virus of the respiratory-nervous disorder has been found to be the cause of a blue-comb condition in chickens which may or may not be associated with respiratory or nervous symptoms would suggest the possible existence of still other

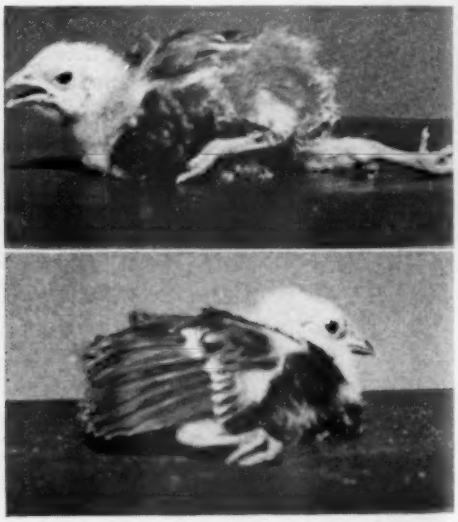

Fig. 2—Artificially infected New Hampshire chicks, 1 month old, showing leg paralysis. The chick (above) with the mouth open, peeped loudly and attempted to get on its feet.

manifestations of the disease. It is conceivable too that the respiratory-nervous disorder which is now known to affect tur-

[2]Jungherr, E., and Levine, J. M.: The pathology of so-called pullet disease. Amer. J. Vet. Res., ii (1941), pp. 261-271.

[3]Brunett, E. L., and Bullis, K. L.: Unknown disease. Proc. 13th Ann. Conf. Lab. Workers in Pullorum Disease (Amherst, Mass., May 1940). Mimeographed.

[4]Report of the Proceedings of the Forty-Fourth Annual Meeting of the United States Live Stock Sanitary Association, pp. 152-153.

[5]Annual Report, 1940-1941, Los Angeles County Live Stock Department, Los Angeles, California, pp. 52-53.

[6]Unpublished data presented in a report received from Doctors E. E. Jones and J. F. Harr,* Poultry Pathological Laboratory, California State Department of Agriculture, Los Angeles, California.

*Now Poultry Disease Specialist, California State Department of Agriculture, Sacramento, California.

key poults in much the same manner as it affects young chickens may manifest itself differently in mature turkeys. For instance, an obviously contagious type of sinusitis of turkeys whose etiology has not been determined, should be studied carefully and consideration given to the possibility of its being related to the respiratory-nervous disorder.

Fig. 4—An artificially infected White Leghorn pullet, 3 months old, showing leg paralysis similar to that seen in fowl paralysis (neurolymphomatosis).

In conclusion, mention must be made of the considerable amount of difficulty the writer has encountered in attempts to transmit the respiratory-nervous disorder by artificial means from adult chickens to other chickens of any age and from chickens to turkeys, or *vice versa*, or from turkeys to other turkeys. This is in contrast to the relative ease with which the disease has been transmitted from affected growing chickens to susceptible chickens of the same age.

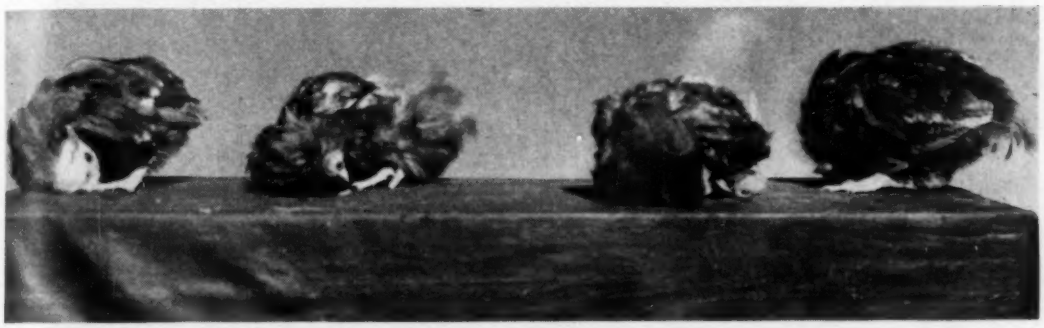

Fig. 3—Field cases of respiratory-nervous disorder, showing peculiar position of the heads. These are New Hampshires, 2 months old.

Ascorbic Acid in Certain Body Fluids of Horses*

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Lexington, Ky.

CERTAIN BODY TISSUES and fluids have been found to contain relatively high concentrations of ascorbic acid, which in some cases are independent of the level in the blood.[1-4] These studies also showed low blood-serum levels of ascorbic acid associated with infections which often reach values encountered in manifest clinical scurvy.

In a study of rheumatoid arthritis, Albany *et al.*[5] suggested that the correlation shown to exist in the severity of the disease, blood sedimentation rate, and the ascorbic acid deficit indicates the importance of the infective factor in many of these cases. They stated that a deficiency of ascorbic acid may be the aggravating factor in the actual production of rheumatic diseases.

In feeding experiments with dairy cattle, Knight *et al.*[6] found that a significant increase in blood-plasma ascorbic acid could be found only by injection methods. Feeding as much as 100 Gm. of ascorbic acid per day for three successive days failed to increase the level of ascorbic acid in the blood.

The observation[7] that a majority of the disorders of the ophthalmotonus have been due to defects in the intraocular fluids deserves consideration in veterinary medicine inasmuch as a decrease in tension of the eyeball is present in acute and chronic cases of periodic ophthalmia. Duke-Elder[7] made a thorough chemical analysis in his study of the intraocular fluids. He elaborated the theory that the intraocular fluid is in thermochemical equilibrium with the blood and is a dialysate of it, the capillary walls acting as the dialysing membrane. The relation of ascorbic acid to the permeability of vessels was recognized by Goldman *et al.*[8] They explained the development of some types of cataracts of the lens as a uveitis which so increases the permeability of the blood-aqueous barrier that the supply of ascorbic acid necessary for metabolism of the lens is decreased and the lens becomes opaque.

Bellows[9] found the ascorbic acid content of the aqueous humor to be about twelve times as much as in blood plasma. He suggested that a diminution of ascorbic acid in the aqueous and lens in cataractous subjects precedes the development of opacity and is not secondary to it.

In our clinical study, we observed a high incidence of cataract in horses which did not have periodic ophthalmia. Most of these cataracts were of a type different from that usually found in periodic ophthalmia, in which entire cataracts are a common sequel and the most common cause of blindness. For these reasons, we have studied the ascorbic acid content of the eye fluids and blood plasma of horses.

A disease of horses (known as wobbles) characterized by incoördination of the hind limbs has been described.[10] It is distinguished by a degeneration of the spinal cord and spinal nerves. In some cases, hemorrhages are found on the arachnoid and within the cord. As a portion of our investigation of the disease, we made ascorbic acid analyses of a number of samples of spinal fluid from normal and diseased animals.

*From the Department of Animal Pathology and the Department of Chemistry, Kentucky Agricultural Experiment Station.

[1]Bouton, S. M. Jr.: Arch. Int. Med., lxiii (May 1939), p. 930.

[2]Wortes, L. J., and Wortes, S. B.: Am. J. Med. Sci., cxcvi (1938), p. 384.

[3]Castex, M., and Schteingart, M.: Presna Medica Argentinia, xxvii (May 1940), p. 905. Abst., J.A.M.A., cxv (July 1940), p. 167.

[4]Faulkner, J. M., and Taylor, F. H. L.: Ann. Int. Med., x (1937), pp. 1867-1873.

[5]Albany, M. A., Harris, L. J., and Ellman, P.: Lancet, ii (1937), pp. 181-183.

[6]Knight, C. A., Dutcher, R. A., Guerrant, N. B., and Bechdel, S. I.: J. Dairy Sci., xxiv (1941), pp. 567-577.

[7]Duke-Elder, W. S.: Physio. Rev., xiv (1934), p. 483.

[8]Goldman, H., and Buschke, W.: Abst., Arch. Ophthalmol., xvi (1936), p. 124.

[9]Bellows, J.: Arch. Ophthalmol., xv (1936), p. 78.

[10]Dimock, W. W., and Errington, B. J.: J.A.V.M.A., xcv (1939), p. 261.

TABLE I—Content of Ascorbic Acid in Blood Plasma After Feeding the Quantities Stated

HORSE NO.	ASCORBIC ACID (GM.)	TIME IN HOURS								
		0	2	4	6	8	10	12	14	24
86	0	0.81*	0.76	0.78	0.81	0.79	.....	.....	.....	.....
93	0	.....	.....	.....	—	0.84	0.84	0.85	0.85	.....
192	0	0.60	0.83	0.79	0.88	0.67	.....	0.93	.....	0.91
51	1	0.71	0.98	0.99	0.71	0.64	.....	.....	.....	.....
22	1	.....	.....	.....	.....	0.74	0.64	0.69	0.69	.....
1	5	0.90	0.92	1.01	0.90	0.98	.....	.....	.....	.....
0	5	.....	.....	.....	.....	1.04	1.06	1.38	.....	0.84
99	10	1.01	1.08	1.20	0.71	0.67	.....	.....	.....	.....
167	10	.....	.....	.....	.....	1.15	1.22	1.28	0.84	.....
195	10	0.59	0.80	0.68	0.85	0.83	.....	0.79	.....	0.82
196	15	0.85	0.800	0.62	0.72	0.87	.....	1.08	.....	0.97
102	20	0.70	0.70	0.71	0.81	0.73	.....	1.00	.....	0.99
197	30	0.60	0.60	0.72	0.86	0.86	.....	0.78	.....	0.92

*Milligrams of ascorbic acid per 100 cc. of plasma.

Phillips *et al.*[11] concluded that the ascorbic acid content of bull semen below 2 mg./100 cc. was associated with impotency, while values greater than normal were often associated with bulls of questionable breeding records. A few samples of horse semen were available for a preliminary study of sterility and are discussed in this report.

METHODS

The samples of eye and spinal fluids were obtained either from the living animal or immediately after euthanasia by chloroform or magnesium sulfate. Semen samples were obtained from the penis of stallions after service.

Ascorbic acid was administered subcutaneously, intravenously, and *per os*. Subcutaneous injections using the buffer suggested by Phillips[11] caused abscesses, as did ascorbic acid in distilled water solution. Therefore, sodium bicarbonate, equal in amount to one half of the ascorbic acid used, was added to the solution in distilled water immediately before making subcutaneous injections. Ascorbic acid in distilled water was used for intravenous injections. For feeding, the ascorbic acid was administered in gelatin capsules.*

In the preliminary work, analyses were made within an hour of sampling. Other than corking the sampling tube, control of light and heat effects was not attempted. No loss in ascorbic acid was found under these conditions in the first hour. Analysis after 24 hours at room temperature, however, showed a 32 per cent loss in ascorbic acid. Samples analyzed in the major part of this study were immediately placed on ice, protected from light and analyzed one to five hours later. No significant loss in ascorbic acid was detected in this procedure.

The method of analysis for ascorbic acid was that of Farmer and Abt[12] in which blood plasma—obtained from oxalated blood—semen, eye and spinal fluids were deproteinized with metaphosphoric acid and the centrifugate titrated with a standardized solution of the oxidation-reduction indicator, 2, 6-dichlorophenolindophenol.

RESULTS—BLOOD PLASMA

The average ascorbic acid content of the blood plasma of 112 normal horses was 0.73 mg./100 cc., with a range of 0.30 to 1.76 mg./100 cc. In 90 per cent of the cases, the ascorbic acid values came within a range of 0.40 to 1.10 mg./100 cc. The changes in the ascorbic acid content of the blood plasma induced by several methods of administration are shown in tables I and II. Two groups of 4 animals each were fed 0, 1, 5, and 10 Gm. of pure ascorbic acid in capsule form. Analyses for ascorbic acid were made at 2-hour intervals up to 8 hours in 1 group and between 8 and 14 hours in the second group (table 1). Quite wide variation in the plasma ascorbic acid

*The ascorbic acid was contributed by Merck & Co., Inc., Rahway, N. J.

[11]Phillips, P. H., Lardy, H. A., Heizer, E. E., and Rupel, I. W.: J. Dairy Sci., xxiii (Sept. 1940), p. 872.

[12]Farmer, C. J., and Abt, A. F.: Proc. Soc. Exp. Biol. & Med., xxxii (1935), p. 1625.

was found which could not be correlated with treatment of the pretreatment level of ascorbic acid in the blood.

A second series of five animals were fed 10, 15, 20, and 30 Gm. of pure ascorbic acid (table I); the fifth animal received no ascorbic acid. Analyses were made at intervals up to 24 hours after treatment. The variation of the blood ascorbic acid of the control animal was nearly as great as that found when gross quantities of pure ascorbic acid were administered in the feed.

INGESTED ASCORBIC ACID NOT ASSIMILATED IN THE HORSE

Variations of plasma ascorbic acid were found to be as great in the individual during the hours following treatment as the variation between individual horses after feeding of comparatively large amounts of ascorbic acid. After a consideration of the increased ascorbic acid content of the blood following subcutaneous and intravenous injection, as mentioned later, as well as the probable experimental error, it would appear that little or none of the vitamin was assimilated when fed. That it is not assimilated in the blood of horses was indicated in another experiment in which one animal received 1 Gm. of ascorbic acid daily for 12 days. The ascorbic acid content of the blood plasma varied from a pretreatment value of 0.90 to 0.93 mg./100 cc. at the end of the 12-day period, with a low value during the course of administration of 0.60 mg./100 cc.

Apparently, this vitamin is not assimilated in horses when administered *per os*. Dairy cows also did not assimilate it when administered in this manner, according to Knight *et al.*[6]

The results of administering ascorbic acid by feeding, and by subcutaneous and intravenous injections are illustrated in table II. Analyses made at hourly intervals showed a maximum of 9.11 mg./100 cc. of plasma one hour after subcutaneous injection of 20 Gm. Intravenous injection of the same quantity raised the level to a maximum in the blood of 7.62 mg./100 cc. after one hour. The administration of 20 Gm. of ascorbic acid in the feed, however, increased the level to only 0.95 mg./100 cc. as a maximum at the end of seven hours. The level in the blood plasma was still above the pretreatment value at the end of 24 hours. Results show that the blood ascorbic acid level following subcutaneous injection remained higher over a longer period than it did after intravenous injection of the same quantity. The rapid fall of ascorbic acid content following intravenous injection is shown in the case where, after a two-hour interval, there was little difference between the effects of a 10-Gm. and a 20-Gm. dose.

TABLE II—Variation of Blood Ascorbic Acid with Administration and Time

Horse No.	Administration	Time in Hours									
		0	1	2	3	4	5	6	7	12	24
192	Intravenous — 10 Gm.	0.73*	3.69	3.56	2.64	2.11	2.14	1.84	.....	.....	1.03
197	Intravenous — 20 Gm.	0.68	7.62	3.58	2.70	2.16	1.93	1.78	.....	.....	0.84
196	Subcutaneous — 20 Gm.	0.66	9.11	6.12	4.37	3.08	2.32	2.03	.....	.....	1.09
102	Fed (capsule) — 20 Gm.	0.63	0.80	0.88	0.91	0.84	0.84	0.90	0.95	0.86	0.76
191	Control	0.76	0.74	0.74	0.75	0.74	0.73	0.73	.....	.....	.....

*Blood plasma ascorbic acid—mg./100 cc.

EYE FLUIDS

In 30 horses with no symptoms of periodic ophthalmia, the average ascorbic acid content of the aqueous humor was 21.91 mg./100 cc., with a range of 6.9 to 34.2 mg./100 cc.

Of six animals with the lowest content of ascorbic acid in the aqueous humor (6.9 to 14.6 mg./100 cc.), five were over 20 years

of age. Four of the five had either vitreous opacities or cataracts which were extensive enough to be classed as an unsoundness. Other animals in this group with a normal ascorbic acid content in the aqueous humor did have opacities in the media of varying density. Although it would not be expected that all opacities of the lens or vitreous are related to ascorbic acid in the eye fluids, there appeared to be a correlation among age, ascorbic acid content of the aqueous, and significant opacities in the media in the animals of this group.

In the eyes of 24 horses with no symptoms of periodic opththalmia, the average ascorbic acid content was 20.41 mg./100 cc. in the vitreous humor. Although the average ascorbic acid level of the vitreous humor was slightly below that of the aqueous, a similar relationship existed among age, opacities of the media, and ascorbic acid content.

An average ascorbic acid content of the aqueous humor of five horses affected with periodic ophthalmia was 9.19 mg./100 cc., with a range of 5.02 to 13.93 mg./100 cc. In two eyes in which there had been recurrent attacks of periodic ophthalmia within recent months, the ascorbic acid level in the aqueous was 5.02 and 7.83 mg./100 cc. These were the lowest of the five recorded cases. The disease was chronic in the three remaining cases. No attacks have occurred within the past two years.

Although eight daily 1-Gm. intravenous injections of ascorbic acid did not build up the level of ascorbic acid in the blood plasma, it increased the content of the aqueous humor in a chronic case of periodic ophthalmia from 8.73 to 17.00 mg./100 cc.

In another case of chronic periodic ophthalmia affecting both eyes, 2.2 cc. of aqueous humor was withdrawn from the right eye. It contained 5.02 mg./100 cc. of ascorbic acid. In 3 2/3 hours the tension of the eyeball had returned to its previous level, although the aqueous was straw colored and contained only 3.08 mg./100 cc. of ascorbic acid. The level in the left eye was 7.83 mg./100 cc. at this time. Ascorbic acid was then administered in daily 1-Gm. subcutaneous doses. In 11 days, the level in the aqueous humor of the left eye had increased to 14.07 mg./100 cc. On the second day following the last withdrawal of aqueous, there was no change in the left eye except a slightly contracted iris. The cornea became cloudy on the third day. This was followed by the formation of hypopyon and a typical acute attack of iridocyclitis during the fourth day. Withdrawal of the aqueous, which in our cases was always followed by a decrease in the ascorbic acid content of the newly formed aqueous humor, was probably a factor in exciting the acute attack of iridocyclitis.

Tension Presymptomatic of Recurrence

We have observed[13] that in some cases of periodic ophthalmia, a prognosis of future attacks can be made by the tension of the eyeball. The greater the decrease in tension following an attack, the sooner recurrent attacks or blindness are apt to occur.

A relationship between the tension of the eyeball and the ascorbic acid content of the eye fluids was indicated in a 25-year-old saddle mare with no history of periodic ophthalmia. Each of her eyes had a considerable amount of vitreous opacities, as is frequently seen in aged animals; the tension of each eye was decreased and the ascorbic acid content of the aqueous humor was 11.6 mg./100 cc. One should remember that frequently in aged horses, a chronic type of periodic ophthalmia occurs with no history of acute recurrent attacks having taken place.[13] Bouton[1] reported a reduction in vitreous opacities following the administration of ascorbic acid in human beings.

From our small number of cases, it is impossible to determine whether a lowered ascorbic acid content of the eye fluids is a factor in the etiology of periodic ophthalmia or simply a result of the inflammation and consequent change in the permeability of the vessels. A change in any one of the three factors—tension of the eyeball, ascorbic acid content of the eye fluids, or per-

[13]Errington, B. J.: J.A.V.M.A., xcviii (1941), p. 115.

TABLE III—Ascorbic Acid Content in Body Fluids of Horses

SAMPLE	CONDITION	NO. OF ANIMALS	ASCORBIC ACID — MG./100 CC.		
			MINIMUM	MAXIMUM	AVERAGE
Blood plasma	All animals	112	0.30	1.76	0.73
Aqueous humor	Normal	30	6.90	34.20	21.91
Vitreous humor	Normal	24	14.55	29.12	20.41
Aqueous humor	Periodic ophthalmia	5	5.02	13.93	9.19
Spinal fluid	Normal	12	0.83	3.25	1.70
Spinal fluid	Wobblers	11	0.65	2.16	1.31
Semen	All animals	20	0.34	2.63	0.86

meability of the local blood vessels—would affect one or both of the other factors. The question is, of course, what is the cause of the primary variation. The course of the symptoms and the recurrent attacks in periodic ophthalmia can be explained by a consideration of the factors mentioned, even though the ascorbic acid supply may not be the primary cause.

SPINAL FLUID

The average ascorbic acid content of the spinal fluid of 12 normal horses was 1.70 mg./100 cc., with a range from 0.083 to 3.25 mg./100 cc. Ascorbic acid in the spinal fluid of eleven wobblers averaged 1.31 mg./100 cc., with a range from 0.065 to 2.16 mg./100 cc.

Although the average quantity of the vitamin in the spinal fluid was lower in wobblers than in normal horses, the spinal fluid of three of the twelve normal animals had an ascorbic acid content as low as the average of the wobblers. The ascorbic acid of the spinal fluid of only one of the wobblers was above the average of the normal group. In so far as we have been able to demonstrate, no infectious agent is involved in this disease.[9] As there are degenerative changes in the spinal cord, the decrease in ascorbic acid shown in some cases may be the result of the degree or stage of the inflammation. It is impossible to estimate the significance of the tendency for a decrease in the ascorbic acid content of the spinal fluid of wobblers.

Castex[3] concluded that the quantitative variations of this vitamin in cerebrospinal fluid in diseases of the central nervous system of man were inconstant and not related to the disease or alterations of the fluid. He found that the ascorbic acid content of cerebrospinal fluid may be normal in the presence of ascorbic acid inadequacy.

SEMEN

In the semen of 20 stallions, the ascorbic acid averaged 0.86 mg./100 cc., with a range from 0.34 to 2.63 mg./100 cc.

No correlation between the level of ascorbic acid in the blood plasma and that in the eye fluids, semen or spinal fluid was observed (table III). One of the semen samples was from a stallion which had become totally sterile. The number of spermatozoa in his semen was somewhat less than normal and the motility of more than half of them was very slight. Another semen sample was from a stallion of low fertility. A few of the mares bred to him the previous season had become pregnant. The former stallion was one of five stallions assembled from different parts of the United States during the previous few months. The latter was one of six stallions on one farm. In each case, there were stallions in the same group whose semen ascorbic acid was higher while others were lower than the two with poor breeding records.

Results of the semen examination of the two stallions of low fertility indicated that there was no deficiency of ascorbic acid that could be related to the low fertility.

SUMMARY

Results of ascorbic acid analyses of the blood plasma, semen, spinal fluid, and the

aqueous and vitreous humors are given.

Horses apparently do not assimilate ascorbic acid when it is administered *per os*.

No correlation was observed between the ascorbic acid content of the blood plasma and that of the other fluids examined.

There is a tendency for a decrease in the ascorbic acid content of the spinal fluid and the eye fluids in local inflammatory processes. In cases of periodic ophthalmia, there was a decrease in the ascorbic acid of the aqueous humor of 58.5 per cent from the average normal.

Interspecies Transmission of Avian Leucosis in Embryos*

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VARIABLE RESULTS have been attained in attempts to transmit avian leucosis from one species to another. Failure to reproduce the "leucotic"† state in heterologous species has been reported by Engelbreth-Holm and Roth-Meyer[1] in the guinea fowl and the pheasant; by Stubbs and Furth[2] in the guinea fowl and pigeons and the latter authors cite Ellerman and Bang with the report that guinea fowl, doves, and turkeys could not be infected by the leucosis virus. Wakamatsu[3] failed to infect canaries; geese, ducks, and turkeys were refractory to the disease as reported by Olson;[4] and Schaaf[5] failed to transfer the disease to ducks, geese, pigeons, parrots, canaries, rabbits, guinea pigs, and mice. Jarmai[6] reported that water-fowl, sparrows, rabbits, mice, and guinea pigs were refractory to the disease, and in another instance[7] he failed to transmit the disease to ducks, geese, a quail, a sparrow-hawk, a peacock, and roentgen-irradiated pigeons.

In some instances a relative susceptibility to the experimental transfer of the leucosis agent has been demonstrated: transient anemic dyscrasias which were interpreted as reactions to the virus were reported by Engelbreth-Holm and Roth-Meyer[1] in 3 of 8 guinea fowl; by Schaaf[5] in 3 of 5 guinea fowl; and by Morelli and Vercellone[8] in 2 of 5 pheasants.

Leucosis has been transmitted from the chicken to other species of birds: to a hybrid pheasant hen by Stubbs;[9] to 7 of 13 turkeys, 3 guinea fowl, and 2 pheasants by Jarmai.[7] In the latter instance the inoculation of leucemic turkey blood into chickens induced an attentuated reaction that became enhanced with repeated serial inoculations into chickens. Greppen[10] produced fatal leucosis in 2 of 3 guinea fowl and in 1 of 3 turkeys inoculated intracerebrally. Glover[11] reports the passage of a strain of leucosis agent, which originated in the turkey, through 16 generations of young chicks.

The spontaneous occurrence of lymphomatous dyscrasias in birds, other than the chicken, which were submitted for routine postmortem examination, has been reported. If the number of reports is evidence of its relative incidence, it is infrequently encountered in birds other than the chicken. Lymphomatous dyscrasias has been reported in the pheasant, Jungherr[12] and Harriss;[13] in the turkey, Downham[14] and Durant;[15] in a small parakeet, Jarmai[16] and in canaries, Kogler[17] and Haupt.[18] Richter[19] has reported lymphomatous dyscrasias in the canary, pigeon, duck, goose, and turkey. The authors[20] have also encountered several cases of visceral lymphomatosis in turkeys, in some instances with concurrent enterohepatitis.

METHODS

Leucemic blood (Strain A) from experimentally infected chicks or chick embryos was inoculated, according to the intravenous technique described by Eichhorn[21] into 10 to 16 day old incubating embryos of the following species: turkey, *Meleagris gallopavo;* duck, *Anas boschas;* guinea fowl, *Numida meleagridis;* pheasant, *Phasianus colchicus;* and quail, *Coturnix communis.* In addition to these, several

*From the Animal Disease Station, Bureau of Animal Industry, U. S. Department of Agriculture.

†The word "leucotic" is used at the request of the author.—ED.

CHART I—Cumulated Data on Transfers of Leucosis in Embryos

SPECIES INOCULATED	PASSAGE	SOURCE	NUMBER INOCULATED	LEUCOSIS		P. I.* DAYS
				NUMBER	PERCENT	
Duck	1	Chick embryo	9	0		
	1	Chick	15	3	20	9.6
	1	Guinea	1	0		
	1	Turkey embryo	25	9	36	6.8
	2	Duck embryo	4	4	100	4
			54	16	29.6	6.8
Turkey	1	Chick	37	36	96.3	8.1
	1	Chick embryo	10	8	80	8.0
	1	Guinea embryo	6	5	83.3	10.6
	2	Turkey embryo	23	19	82.5	6.9
	3	Turkey embryo	10	9	90	10.6
			86	77	89.5	8.8
Guinea fowl	1	Turkey embryo	9	7	77.7	7
	1	Chick embryo	2	2	100	5
	1	Chick	11	10	90	6.2
	2	Guinea embryo	4	4	100	6.6
			26	23	88.5	6.2
Pheasant	1	Chick	3	2	66.7	8
	1	Turkey embryo	2	2	100	7.5
	1	Guinea embryo	4	4	100	6.5
			9	8	88.8	7.3
Quail	1	Chick embryo	4	3	75	5.75
	1	Turkey embryo	4	4	100	4.2
	1	Pheasant embryo	3	3	100	6.3
			11	10	90.9	5.41

*"P. I.," the post-inoculation period refers to the life span of the bird from the time of inoculation

11 to 12 day old incubating chick embryos were inoculated as controls. One-to-6 day old, hatched-out turkeys, ducks, and quail were also inoculated with leucemic blood from the same sources. Blood smears from small allantoic veins were taken from the inoculated embryos so that the development of the disease could be observed. When the embryo was considered suitably affected, blood was withdrawn from a large allantoic blood vessel for transfer to both homologous and heterologous species of embryos and hatched-out chicks. Transfers in "zig-zag" fashion, from chick to turkey and *vice versa*, were also made.

Pure blood was collected and transferred immediately, or sodium citrate was used as the anticoagulant. Bacteriological sterility was checked on each occasion.

All birds which were inoculated and which succumbed were examined as soon as possible after death. Impression smears were made of the liver, spleen, and kidneys and with some embryos the same tissues were fixed in ten per cent formalin or Bouin's solution for sectioning. The tissue impression smears and the blood smears were subsequently stained by Wright's stain or by combined Wright-Giemsa's stain for microscopic examination. The fixed material was imbedded in paraffin, sectioned, and stained with hematoxylin and eosin.

RESULTS

Eighty-six turkey embryos were inoculated with blood from chick, turkey or guinea fowl embryos, or hatched-out chicks, *Gallus domesticus*, of which 77 (89.5%) developed the leucemic dyscrasia and died after an average post-inculation period of 8.8 days. Of these, 53 were inoculated as first generation turkey passages in which case the disease was induced in 49 (92.4%) with death at an average of 9.2 days. Serial passages of blood from the above embryos

elicited a leucemic response in 19 of the 23 that were inoculated (82.6%) with death after an average period of 6.9 days. Third generation passages of the agent induced the disease in 9 of the 10 that were inoculated (90%) with death after an average period of 10.6 days. Data for all inoculations are recorded in chart I.

Fifty-four duck embryos were inoculated with blood from leucotic chick, turkey, guinea-fowl, and duck embryos or from

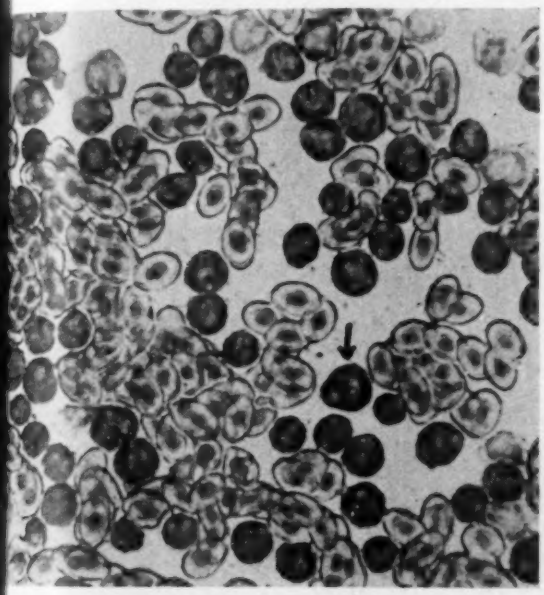

Fig. 1. Blood smear from "leucotic" chick embryo (635-5) showing numerous hemocytoblasts. Wright's-Giemsa Stain. X640.

hatched-out "leucotic" chicks. Sixteen (29.6%) developed leucosis and died after an average period of 6.8 days. Fifty of these embryos were first generation transfers of which 12 (24%) developed fatal leucosis after an average of 8.2 days. All of the four embryos which were inoculated with second generation material developed the dyscrasia after an average of 4 days. In two instances a markedly enhanced reaction was induced in first passage duck embryos in an average of 5.5 days, and in a second generation transfer in 4 days.

Of the 26 guinea fowl embryos which were inoculated with "leucotic" blood from chicken, turkey, or guinea embryos, and from hatched-out chicks, 23 (88.46%) developed leucosis and died after an average period of 6.2 days. Twenty-two constituted first passages and 4, second passages, of which 19 (86.4%) and 4 (100%), respectively, developed leucosis after average periods of 6 and 6.6 days.

Leucosis and death were induced on primary passages in 10 of the 11 quail embryos which were inoculated (90.9%) after an average period of 5.41 days.

Eight (88.8%) of the 9 pheasant embryos which were inoculated with first passage leucosis material succumbed to leucosis after an average period of 7.3 days.

The initial hematological response which was indicative of the disease in these various species was the appearance of numerous polychromic erythrocytes and erythroblasts in the circulation. Subsequently, hemocytoblasts appeared in progressively

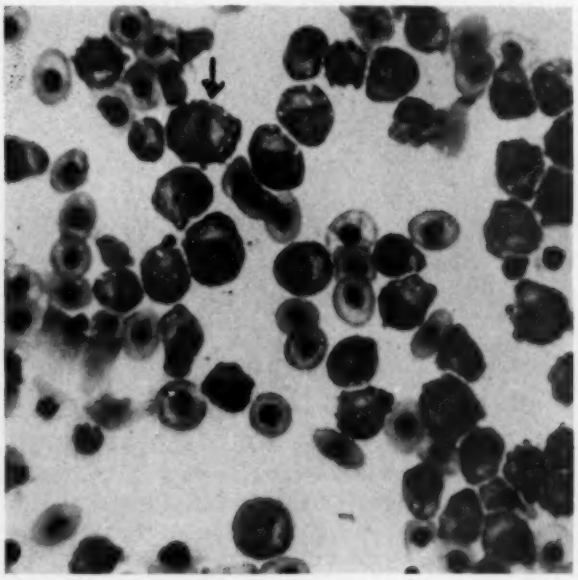

Fig. 2. Blood smear from "leucotic" guinea fowl embryo. Wright's-Giemsa Stain. X640.

increasing numbers, and when these anaplastic cells became relatively numerous in the circulation (fig. 1 to 6), the embryos became moribund and died. Those cases in which the disease assumed a very acute manifestation exhibited some cells in the circulation in mitotic division and some which possessed morphological characteristics of the mesenchymal cell.

Gross pathological changes in the heterologous species of embryos were confined to the liver, spleen, and kidneys, as was the case with the leucotic chick embryo. In the majority of the homologous and heterologous embryos, which were subsequently diagnosed as leucotic by microscopic examination of the tissue smears, the livers were enlarged, turgid, and red. When the embryos were exsanguinated, the liver appeared pale-green. The spleens of most of

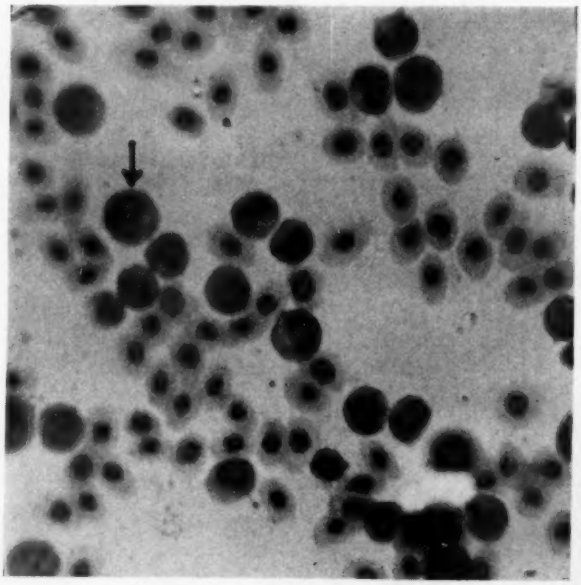

Fig. 3. Blood smear from "leucotic" pheasant embryo. Wright's-Giemsa Stain. X640.

the positive cases were enlarged, friable, and frequently cherry-red. The kidneys were swollen and red, and when exsanguinated these appeared, as with the liver, pale-green.

The tissue impression smears of the affected livers, spleens, and kidneys demonstrated numerous immature lymphoid cells (hemocytoblasts) when examined microscopically. Tissue sections from the leucosis-affected turkey embryos showed extensive intra- and extravascular infiltrations of the livers and kidneys with these anaplastic cells. The normal architecture of these organs was masked to some extent by the extensive leucemic infiltrations; in some cases the parenchymatous tissue was partly or entirely displaced by "leucotic" foci.

Chick embryos which were inoculated as controls for the above turkey, duck, guinea fowl, pheasant, and quail embryos demonstrated hematological responses and pathological tissue changes similar to those which were observed in the various heterologous species. The incidence of leucosis among the chick embryos which paralleled the first turkey passage was 83.2 per cent; the second turkey passage, 70.3 per cent; and the third, 12.5 per cent. The post-inoculation periods averaged 7.9, 8.25, and 10 days, respectively. Control inoculation of chick embryos for the first and second passages in duck embryos resulted in incidences of 73.6 per cent and 88.8 per cent, respectively, with average post-inoculation periods of 7.3 and 3.75 days. Twenty of 28 susceptible chicks (71.4%) that were

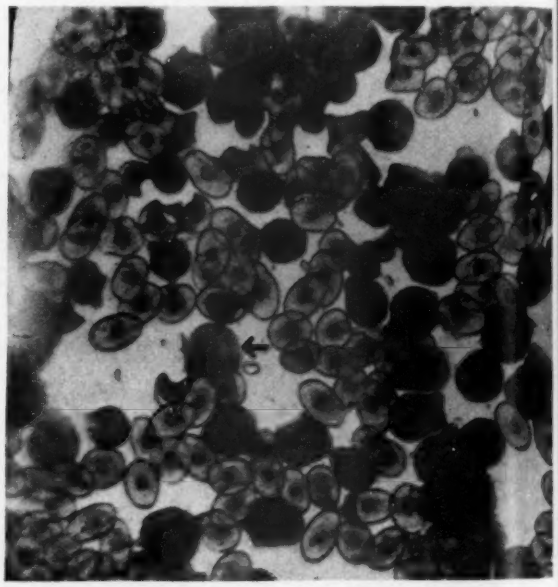

Fig. 4. Blood smear from "leucotic" turkey embryo. Wright's-Giemsa Stain. X640.

inoculated with leucosis-affected turkey embryo blood developed leucosis and died after an average period of 16.8 days. Twenty-three, 2-day-old poults, similarly inoculated, died following the appearance of numerous polychromic erythrocytes and erythroblasts in the blood after an average period of 16.5 days. Two of the latter exhibited lesions of leucosis at autopsy, and of the remainder, 5 were suspicous and 16 showed

CHART II—Data on Transfer of Leucosis to Heterologous Chicks

Species Inoculated	Passage	Source	Number Inoculated	Leucosis		P. I.* Days
				Number	Percent	
Poults	1	Turkey embryo	23	2	8.7	16.5
		Chick embryo	7	0	0	
		Chick	27	11	40.7	5.9
			57	13	22.8	11.2
Duck	1	Duck embryo	15	0	0	
	1	Chick	4	0	0	
			19	0	0	
Pheasant	1	Chick embryo	1	1	100	9

*"P. I.," the post-inoculation period refers to the life span of the bird from the time of inoculation to death.

neither gross nor microscopic lesions. No leucosis was induced in the seven poults which were inoculated with affected chick embryo blood, but when 27 poults were inoculated with "leucotic" chick blood, 11 (40.7%) developed the disease and died after an average period of 5.9 days. Four-day-old hatched-out ducklings were entirely refractory to the disease; of the 19 which were inoculated with blood from chicks and duck embryos, none developed leucosis. The one, 1-day-old, hatched-out pheasant, which was inoculated, developed fatal leucosis, 9 days after inoculation. (See chart II.)

Discussion

The incidence of the disease among the inoculated chick embryos may be inaccurate due to the number of degenerated embryos (18%) resulting from excessive exposure to the incubator temperature. It is possible that a greater percentage of duck embryos succumbed to the disease than is reported since 60 per cent of the total number inoculated were degenerated at autopsy examination.

The virulence of the leucosis agent was little affected by passage into turkey, guinea fowl, quail, and pheasant embryos; the reason for its marked exacerbation, when passed successfully through two series of duck embryos, is as yet speculative. The post-inoculation period in quail embryos was relatively short, but this might be attributed to the small size of the embryo, the dosages for all of these species being between 0.05 cc. and 0.1 cc. of "leucotic" blood. "Zig-zag" passages from the chick embryo to the turkey embryo and then back to the chick embryo, even when repeated several times, induced no marked alteration in the character of the "leucotic" response. There was no attentuation of virulence when passed through turkeys as was reported by Jarmai.[16]

The question of whether these experiments constitute heteroplastic transplanatations of anaplastic tissue or the transfer of an autonomous blastogenic agent remains unanswered, since few transfers have thus far been attempted into embryos with cell-free inoculums. The similarity among these species of the organs involved and of the character of the lesions raises the question whether these hemocytoblastic cells, which are pathognostic of the disease, are of chick origin or whether they originate in every one of the heterologous embryos which developed the leucemic dyscrasia. The disease has been transferred *in ovo* from the chick to the turkey and from the turkey to the duck, guinea fowl, and quail. It has similarly been transferred from chicks to pheasants and from the latter species to quail; and from chicks to guinea fowls and from the latter to pheasants. It

has been transferred through three generations in turkey embryos, two in ducks, and two in the guinea fowl embryos. Leucosis was induced in 22.8 per cent of 57 turkey poults and in one pheasant chick which were inoculated. Jarmai[7] theorizes that every class of birds has its own strain of leucosis. In this case, leucosis has been induced in five different species of birds by the same agent. The type of hematologic response in the heterologous species, which is similar to that induced in the chick embryo, suggests the presence in these heterologous species of a potential leucemia apparatus, with susceptibilities similar to those of the chick.

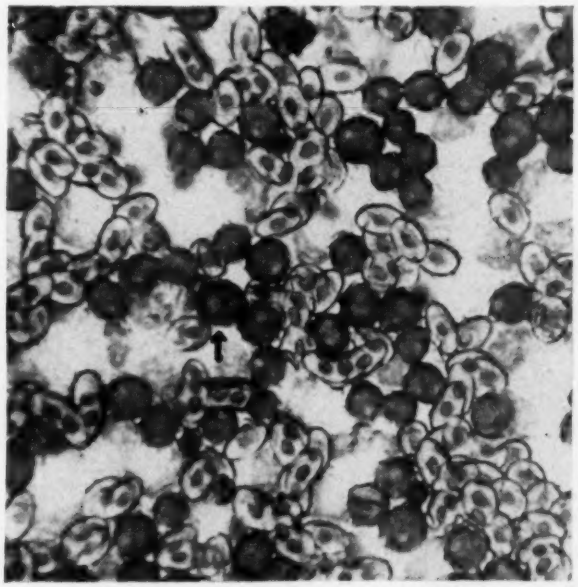

Fig. 5. Blood smear from "leucotic" quail embryo. Wright's-Giemsa Stain. X640.

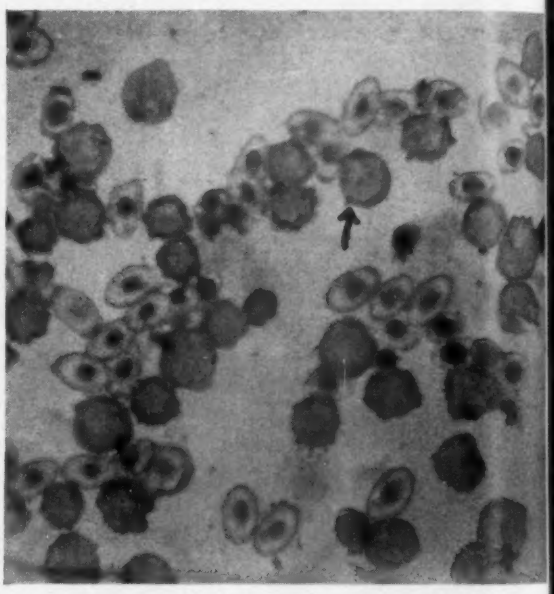

Fig. 6. Blood smear from "leucotic" duck embryo. Wright's-Giemsa Stain. X640.

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Sporadic Bovine Encephalomyelitis: Filtration of the Causal Agent*

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AN INFECTIOUS DISEASE of cattle which has been tentatively termed sporadic bovine encephalomyelitis was first reported in Iowa by McNutt[1] and McNutt and Waller.[2] Since that time, it has been recognized by Boughton[3] in Texas and by Karlson[4] in Minnesota. It is an infectious encephalomyelitis characterized in the late stages by a serofibrinous peritonitis and pleurisy.

The main symptoms are loss of appetite, inactivity, and high temperature; in some cases the animals become prostrate. The principal nervous symptom is a pronounced depression that develops gradually. Since the last report, additional clinical manifestations have been observed. Although the disease usually occurs in animals under 3 years of age, recently it was observed in aged cattle on a single farm. In this instance, the usual encephalomyelitis was not produced in a majority of cases. Of 30 cattle on the farm, 9 were affected and only 2 of these developed encephalomyelitis and died; one was a 9-year-old cow and the other a calf. All of the affected animals first showed tenderness of the feet with swelling just above the hoofs. There was only a slight elevation of temperature at this stage. In 7 cases, these symptoms gradually subsided and the animals made an uneventful recovery, while the other 2 animals developed typical symptoms and lesions. Lameness similar to the above has been observed in only one out of over 40 artifically infected calves. A detailed description of the disease and other information may be found in previous reports.[1, 2, 5, 6]

Additional information was obtained on immunity and resistance. Animals tested several months after recovery were immune. One heifer was solidly immune when tested 14 months after an attack. With one possible exception, all calves were susceptible to the first exposure. The serums from recovered animals neutralized the causal agent. In neutralization tests, a constant volume of sterile serum was added to the same volume of various concentrations of tissue suspensions. The causal agent was regularly neutralized in 1:50 suspensions of infectious tissues and sometimes neutralized in 1:10 suspensions. Intradermal inoculation of the fully virulent infectious agent in guinea pigs produced no apparent disease but resulted in solid immunity in all trials conducted.

Since examinations for the presence of bacteria, pleuropneumonia-like organisms, and rickettsia yielded negative results, efforts were made to filter the causal agent. The results are reported in this paper.

MATERIALS AND METHODS

The procedure followed in most of the filtrations had as its starting material either known infectious tissues and exudates from affected animals or the yolk material and embryonic fluids from infected chick embryos. Tissues, usually liver and spleen, were finely ground in a mortar with sterile physiologic saline. The tissue suspension or the egg material was filtered once or twice through packed, washed, filter-paper pulp, then after the addition of test organisms (*Salmonella pullorum* was used in filtrations 1 to 28, inclusive; in other filtrations *Serratia marcescens* was employed) passed through Berkefeld candles of N or V grade under various

*From the Veterinary Research Institute, Iowa State College; presented before the Section on Research at the seventy-eighth annual meeting of the American Veterinary Medical Association, Indianapolis, Ind., August 11-15, 1941.

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TABLE I—Filtration of the Causal Agent of Sporadic Bovine Encephalomyelitis

FILTRATE NO.	GRADE OF BERKEFELD	FILTRATION PRESSURE (MM. HG)	SOURCE OF MATERIAL	TEST ANIMALS	RESULTS	IMMUNITY TESTS OF SURVIVING ANIMALS
1	V	<508	G. P.	3 G. P.	Negative	Nonimmune
2		<508	Calf	3 G. P.	Negative	Nonimmune
3	V	<508	Calf	6 G. P.	Positive	See table II
4	V	380	G. P.	1 calf	Positive	
5		<508	Calf	1 calf	Negative	Nonimmune
6		<508	Calf and G. P.	1 calf and 6 G. P.	Positive	
7		<508	Calf	1 calf	Positive	
8	N	<508	G. P.	5 G. P.	Negative	Nonimmune
9	N	<508	Calf	1 calf and 3 G. P.	Negative	Nonimmune
10		<508	Calf	1 calf	Negative	
11	N	508	Calf	1 calf	Negative	Nonimmune
12	N	350	C. E.	6 G. P.	Positive	Immune
13	V	<508	C. E.	3 G. P.	Negative	
14	N	350	C. E.	4 G. P.	Positive	Immune
15	N	225	C. E.	6 G. P.	Positive	
16	V	380	C. E.	3 G. P.	Negative	
17	N	580	C. E.	3 G. P.	Positive	
18	N	225	C. E.	6 G. P.	Positive	
19	N	<508	C. E.	3 G. P.	Negative	
20	N	508	C. E.	2 G. P.	Positive	
21	N	250	C. E.	6 G. P.	Negtive	Nonimmune
22	N	250	C. E.	5 G. P.	Negative	Nonimmune
23	N	300	C. E.	6 G. P.	Positive	
24	N	300	C. E.	6 G. P.	Positive	
25	N	180	C. E.	3 G. P.	Positive	2 nonimmune
25A	N	635	C. E.	3 G. P.	Positive	Immune
26	N	110	C. E.	3 G. P.	Negative	Nonimmune
27	N	110	C. E.	3 G. P.	Positive	
28	N	70	C. E.	3 G. P.	Positive	
29	V	80	C. E.	3 G. P.	Negative	Nonimmune
30	V	80	C. E.	3 G. P.	Negative	Nonimmune
31	V	70	C. E.	3 G. P.	Questionable	2 nonimmune, 1 immune
32	V	70	C. E.	3 G. P.	Questionable	1 nonimmune, 1 immune
33	V	70	C. E.	3 G. P.	Questionable	2 nonimmune, 1 immune
34	V	80	C. E.	3 G. P.	Negative	Nonimmune

G.P. = Guinea pig. C.E. = Chick embryo.

conditions. Usually, the candles were prepared and tested by procedures recommended by Mudd[7] or McIntosh and Bedson.[8] After passage of the egg material through the packed paper pulp, it was passed through candles undiluted or diluted 1:20 with sterile physiologic saline, sterile M/15 phosphate buffer at pH 7.6 or pH 6.5, or with Hartley's[9] broth at pH 7.6. All filtrates were inoculated onto agar slants and incubated for one week to test for bacterial sterility. Usually, filtrates were kept in the refrigerator overnight and then inoculated into test animals and/or 6-, 7-, or 8-day-old chick embryos.

Inoculations were made subcutaneously in calves, intraperitoneally in guinea pigs and in the yolk sac in chick embryos. Results were considered positive if some or all of the inoculated animals showed symptoms or lesions of the disease. As an added proof, the infection usually was carried to other susceptible animals and, where possible, the surviving original animals were tested for immunity by reinoculation with known infectious material. Further technical details will be found in the experimental section.

RESULTS

In table I are presented the results of filtration trials in which 34 bacteria-free filtrates were obtained. Filtrates 1 to 24, inclusive, were obtained with tissue suspensions and undiluted egg material passed through Berkefeld candles at pressures less than 508 mm. of Hg except filtrations 17 and 25A, which were effected at pressures of 580 and 635 mm., respectively. Table I shows some omissions as to the grade of the candle. It is known that most, if not all, of these were the medium or N grade.

One half of the filtrates of the first 24 trials were found to contain the infectious agent. Since these results indicated, qualitatively, that only small amounts of the infectious agent were obtained in the filtrates, it appeared desirable to measure, quantitatively, the concentration of the infectious agent in the original material and in the filtrates in order to estimate the loss of the agent during filtration. Assays were made with chick embryos as subjects upon which tests were made. In most cases, it was found that unfiltered infectious yolk and embryonic fluids diluted with yolk and embryonic fluids from uninfected embryos gave positive results, in inoculations of 0.2

cc. of a 10^{-8} dilution, as indicated by death of the embryos in from 3 to 8 days and successful passage of the agent from these dead embryos through additional embryos. In some cases, embryos inoculated with a 1:1,000,000,000 dilution died, indicating that the limit of dilution had been approached.

Although embryos are killed by high dilutions of the causal agent, guinea pigs were found to be superior to the embryos for testing filtrates for the presence of the agent. Some filtrates from undiluted infectious embryonic material kill all inoculated embryos, but usually only a few or none at all are affected. In many cases where no "takes" from filtrates inoculated into embryos were observed, tests with guinea pigs gave positive results; however, the guinea pigs were given 1-cc. inoculations whereas the embryos usually were given 0.2-cc. inoculations. It may be that the guinea pig requires less of the infectious agent for a "take" than the chick embryo. In no case was it possible to demonstrate the agent in filtrates that gave negative results in guinea pigs. Assays of material before and after the preliminary filtration through paper pulp indicated that only very small losses of the agent occur during that process.

Since these preliminary attempts at filtration yielded unsatisfactory concentrations of infectious agent, the usual procedure was modified in obtaining filtrates 25 through 34, except for filtrate 25A. In these trials, infectious embryonic material was diluted 1:20 with various diluents after passage through the paper pulp; then it was passed through candles. Assays were made in embryos before and after filtration. In filtration 25, the material was diluted with sterile physiologic saline. The reaction of this dilution and the original material was pH 8.3 as determined with the glass electrode. Following the collection of filtrate 25, the same starting material, undiluted, was passed through the same filter without any further manipulation of the candle to obtain filtrate 25A. Filtrate 26 was similarly obtained, with M/15 phosphate buffer at pH 7.6 as the diluent. Filtrates 27 through 34 were obtained as above with Hartley's broth—the broth recommended in ultrafiltration studies by Elford[10]—at pH 7.6, except filtrates 31 and 32, in which the reaction was pH 6.5.

Because the addition of a test organism to the material before filtration is open to the criticism that one more variable is added to an already complex system, filtrations 28 through 34 were carried out in the following manner: After the material was diluted with the broth, it was passed through the candle fractionating the filtrate as collected into three or four 10-cc. portions; then the test organisms were added to the unfiltered material and the filtration and fractionation again carried out under the same conditions as to temperature and pressure. Dilutions of the second or third fraction of the filtrates obtained before and after the addition of test organisms were inoculated into embryos. The second or third fraction collected after the addition of the test organism also was inoculated into guinea pigs. The bacterial test of the candle was considered satisfactory if no organisms appeared in the fractions used for inoculations after one week of incubation.

Since there was an indication that the agent is unstable out of contact with living tissues, filtrations 33 and 34 were effected in the manner described and inoculations were made into embryos and guinea pigs as soon as possible after filtration. Thus, only about six hours elapsed from the time of removal of the material from the infected embryos until filtrations were completed and inoculations made.

The results show that in no case were amounts of causal agent present in the filtrates sufficient for satisfactory assay with chick embryos. In only a few cases was an occasional embryo killed by inoculation of filtrates 25 through 34 (1:20 dilution of the starting material). Thus, it is evident

[9]McIntosh, J., and Bedson, S. P.: A System of Bacteriology in Relation to Medicine (His Majesty's Stationery Office, London, 1931), ix, p. 118.

[9]Hartley, P.: The value of Douglas' medium for the production of diphtheria toxin. J. Path. a Bact., xxv (1922), p. 479.

[10]Elford, W. J.: Handbuch der Virusforschung (Julius Springer, Vienna, 1938), i, p. 156.

TABLE II—Effect of the Addition of a Suspension of Normal Guinea Pig Liver and Spleen to a Filtrate

Infectivity Tests 12/14/39			Immunity Tests 1/16/40
No. of Animals	Inoculum	Results	
3 guinea pigs	1 cc. unfiltered material	3 sick, 1 survivor	Immune, no reaction
3 guinea pigs	1 cc. filtrate	No reaction, 3 survivors	Nonimmune, no survivors
3 guinea pigs	1 cc. filtrate + 0.5 cc. liver and spleen susp.	3 sick, 2 survivors	Immune, no reaction
3 guinea pigs	1 cc. liver and spleen susp.	No reaction, 3 survivors	Nonimmune, no survivors

that filtration under the conditions employed results in an almost complete loss of the causal agent. Whether a filtrate was positive or negative could be determined only by inoculations in guinea pigs.

Although the results obtained from filtrates 31, 32, and 33 were designated as questionable since no symptoms of the disease were observed in the inoculated guinea pigs and since only one animal in each group upon inoculation with a challenging dose of infectious agent showed immunity, it seems probable that a small amount of the agent may have been present in these filtrates. In connection with this, it may be mentioned that several hundred guinea pigs were inoculated with this infectious agent without finding a single one immune unless due to intentional exposure to the disease.

As mentioned above, it would appear that the agent is unstable out of contact with living tissues, especially in filtrates. It has been observed that calf-brain tissue and some relatively cell-free exudates do not produce symptoms as quickly or as severely as when they contain suspended liver or spleen tissues. To ascertain if the addition of a suspension of normal guinea pig liver and spleen would serve to increase the ability of the agent in filtrates to infect, or in some way to make the agent more stable, the following experiment was performed: The infectious material and filtrate were those obtained in filtration 3. Guinea pigs were inoculated with the filtrate with and without the addition of a saline suspension of normal guinea pig liver and spleen, and with the unfiltered material as controls. Controls also were included with the liver and spleen suspension. Thirty-three days later, surviving animals were tested for immunity by inoculations of fresh infectious material. The data are shown in table II.

The results appear to indicate that the liver and spleen of the normal guinea pig help to obtain a "take" when filtrates are inoculated. It is apparent that if this experiment were repeated, it would be improbable that the same clear-cut results would be obtained, due to the fact that the number of animals used is small and the concentration of the agent in the filtrate would necessarily be within a certain range.

Work is being continued toward devising suitable technics for obtaining filtrates containing higher concentrations of the causal agent. It is planned to use other types of bacterial filters, especially the scintered glass type, to ascertain if adsorption or inactivation of the agent occurs during filtration. The stability of the agent is being further investigated.

SUMMARY

Results obtained in studies on the filtration of the agent of sporadic bovine encephalomyelitis are presented. Of 34 filtration trials with Berkefeld N or V candles, 15 filtrates were found to contain sufficient agent to infect guinea pigs, 3 were questionable, and the remainder were negative.

Assays in chick embryos before and after filtration indicated an almost complete loss of the causal agent during filtration. Assays

of unfiltered material killed embryos in dilutions as high as 1:1,000,000,000.

Evidence is presented that the addition of suspensions of liver and spleen of the normal guinea pig to filtrates increases the possibility of infecting guinea pigs with filtrates of the causal agent.

Further lines of investigation are indicated.

ADDENDUM

Since this paper was written, a coöperative study with Dr. Alfred M. Lucas of the Zoölogy Department, Iowa State College, revealed very small, coccus-like bodies in and about the cells of certain tissues. Should these bodies prove to be the active agent, their size would perhaps account for the difficulties of filtration.

Intestinal Cultures For Detecting Salmonellosis in Young Turkeys*

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OUTBREAKS of paratyphoid infections are frequently responsible for severe mortality in poults. When specimens of chicks and poults are submitted to this laboratory for examination, the diagnostic procedure does not usually include cultures from the intestinal tract. This is probably true of many laboratories engaged in diagnosing poultry diseases. In a brief search of the literature describing outbreaks of salmonellosis in these animals, no cases were found in which the writers reported culturing the intestine. The present study was undertaken to determine the advisability of including such cultures in the routine bacteriological examination of turkey poults.

Specimens from 100 lots of poults received for diagnosis between Apr. 24 and Nov. 30, 1941, were examined by the "routine" method, and also by the "intestinal" method. These procedures are described below. Each of the 366 young turkeys submitted were examined in this manner. Bacteria belonging to the Salmonella group were recovered from 40 lots involving 164 specimens, 112 of which actually harbored the organisms.

METHODS

Routine Cultures—In our laboratory, beef extract agar cultures are routinely made from the hearts and livers of all cases; from the lungs, gizzards, yolks, and other organs when lesions or symptoms so indicate; and from the bone marrow of specimens undergoing decomposition. The above described procedure will hereafter be referred to as the "routine" method. Basically, it corresponds to the official method of the Conference of Official Research Workers in Animal Diseases of North America for the diagnosis of pullorum disease in young chicks.[1]

Intestinal Cultures.—Intestinal tracts were removed from the specimens and laid open with a pair of flamed scissors. Several loopfuls of small intestinal and cecal contents, including scrapings from the mucosa, were added to tubes of tetrathionate broth (Difco). After enrichment by incubating for 15 to 24 hours, a loopful of this growth was streaked on plates of MacConkey's agar, Difco. Following overnight incubation, nonlactose-fermenting colonies were picked for study. In a few instances phenol-red-lactose agar was used as the differential medium, but MacConkey's medium was found to be more satisfactory.

In the first 12 lots examined parallel cultures were made, using the enrichment procedure described above and direct streaking of intestinal material on MacConkey's agar plates. The direct method yielded Salmonella organisms from three birds in two lots, while the tetrathionate enrichment method was positive in eight birds from

*From California State Department of Agriculture, Poultry Pathological Laboratory, Los Angeles.

[1]Report of the Conference of Official Research Workers in Animal Diseases of North America on Standard Methods of Pullorum Disease in Barnyard Fowl. J.A.V.M.A. lxxii, (1933), p. 487.

three lots. The enrichment method was, therefore, adopted for the remainder of the study.

RESULTS

Organisms Isolated.—*Salmonella pullorum* was isolated more frequently than any other member of the genus. It was encountered in 22 lots of poults. Two of the strains fermented maltose.

Eleven varieties of paratyphoid organisms were recovered from the 100 lots examined. Classified antigenically according to the Kauffmann-White schema,[2] the following species were found to be involved: *Salmonella typhimurium* (5 lots), *S. anatum* (4 lots), *S. panama* (2 lots), *S. bareilly, S. bredeney, S. derby, S. give, S. kentucky, S. montevideo, S. san diego,* and *S. senftenberg* (1 lot each).

Comparison of Methods for Isolating S. Pullorum.—The data show that culturing the hearts, livers, and such other organs as the particular circumstances warrant—the "routine" methods—was a satisfactory procedure for the isolation of *S. pullorum.*

Seventy-one poults, involving 22 outbreaks, were found to harbor this organism. In 13 of these birds, only the cultures from the intestine were positive, but positive cultures were obtained by the "routine" method from at least one bird in each lot.

In 10 lots, and in 38 birds, cultures from the intestines were negative, and pullorum disease organisms were recovered only by the "routine" metho l. In 12 lots, and in 20 birds, both "routine" and intestinal cultures were positive. These results are summarized in table I.

TABLE I.—Salmonella Pullorum Infections, Showing Positive Lots and Birds and the Method of Isolation.

	Pullorum disease positive cultures obtained by:			
	Intestinal Method Only	Routine Method Only	Both Methods	Total
No. of lots	0	10	12	22
No. of birds	13	38	20	71

[2]This work was done by P. R. Edwards, Department of Animal Pathology, University of Kentucky.

Comparison of Methods for Isolating Paratyphoid Organisms.—For detecting paratyphoid infections the "routine" method of culture was found to be inadequate. Paratyphoid infection was diagnosed in 21 lots of poults, based upon the isolation of paratyphoid organisms from 42 birds. Eight of these lots yielded paratyphoid organisms solely from the intestines. In four lots only the routine cultures were positive, while in nine additional lots, both "routine" and intestinal cultures were positive. Likewise, 19 of the 42 poults harboring paratyphoid bacteria were detected solely by intestinal culture, "routine" cultures being negative. Table II summarizes these results.

TABLE II.—Paratyphoid Infections, Showing Positive Lots and Birds and the Method of Isolation.

	Paratyphoid infection positive cultures obtained by:			
	Intestinal Method Only	Routine Method Only	Both Methods	Total
No. of lots	8	4	9	21
No. of birds	19	5	18	42

The paratyphoid positive lots detected are summarized in table III according to the number of birds examined in each lot. It will be seen that culturing a larger number of birds did not increase the relative frequency of paratyphoid isolations by the the "routine" method. In 12 lots, and "routine" method. On the other hand, over one-half of the positive lots with four or more specimens was missed by this method.

DISCUSSION

The results of this study show that culturing the intestines of young turkeys will materially increase the frequency of Salmonella isolations. Two questions must be considered, however, before deciding to

adopt this somewhat troublesome method as a routine procedure.

The first question relates to its practical value. In the diagnosis of pullorum disease, the use of intestinal cultures does not seem to be of great importance. This infection is almost always septicemic in young birds, and the causative agent should be readily recovered from the heart blood, liver, and lungs. None of the cases of pullorum disease described in this paper would have been entirely missed if the intestine had not been cultured, and in only one case was the margin dangerously close. In this instance, 14 birds were examined, only one of which yielded the organism by "routine" culture. Seven of these birds were found to harbor *S. pullorum* in the intestine.

TABLE III.—Relation of Number of Specimens Examined to Detection of Paratyphoid Infection.

No. of Specimens in Each Lot	Paratyphoid cultures: obtained by:			Total
	Intestinal Method Only	Routine Method Only	Both Methods	
3 or less	1	4	3	8
4 or more	7	0	6	13

For detecting cases involving paratyphoid organisms, intestinal cultures are decidedly helpful. More than one-third of these lots of poults would have been missed if such cultures had been omitted, as would nearly one-half of the affected birds, as shown in table II.

The second question involves the significance of those instances in which Salmonella organisms are recovered from the intestine. Are these cases truly infections, or are the organisms present in the intestine without causing damage?

The *S. pullorum* recoveries apparently represented actual outbreaks since the organisms were recovered from organs other than the intestine in each instance.

Paratyphoid organisms were recovered from the intestines in seventeen lots, nine of which were in conjunction with recoveries from other organs. These nine lots evidently represented true septicemic infections. The remaining eight lots, in which only intestinal isolations were accomplished, involved heavy mortality in the broods. In four of these instances, no intercurrent infections or other causes for the mortality could be found. This would indicate that a significant proportion of the cases in which paratyphoid organisms are isolated exclusively from the intestines may represent actual disease outbreaks.

From the foregoing, it appears that the routine bacteriological examination of poults should include cultures made from the intestines.

SUMMARY

1. One hundred lots totaling 366 young turkeys were cultured, and the isolations of Salmonella organisms from the intestines were compared to those from other organs. Forty lots and 112 birds were positive.

2. Eleven species of Salmonella other than *S. pullorum* were isolated.

3. Pullorum disease was not detected by intestinal cultures and missed by the remaining cultures in any one lot.

4. Paratyphoid infection was detected by intestinal cultures and missed by the remaining cultures in one-third of the lots.

5. It is concluded that instances in which paratyphoid organisms are recovered solely from the intestinal tracts may represent true infections. Routine examination of young turkeys should include intestinal cultures.

ACKNOWLEDGMENTS

The work of E. E. Jones and J. F. Harr, of this department, and R. A. Mueller, of the Los Angeles County Live Stock Department, in conducting postmortem examinations is acknowledged, and also that of P. R. Edwards of the University of Kentucky for the antigenic analyses of the Salmonella species.

Further Studies on the Histology of the Bovine Kidney*

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IN A PREVIOUS article,[1] some of the anatomic characteristics of bovine kidneys were discussed, such as the lobation of the kidneys, the approximate location of the various segments of the nephron in the cortex and medulla, the comparative size of each segment, and the number of tubules found in a given medullary ray of a lobule. Sisson[2] has given a very clear description of the gross characteristics; consequently, these will not be discussed here.

The thickness of the cortex and medulla of calves at birth is found to vary from 14-19 mm. and the thickness of the cortex is 3-6 mm., while at nine years of age the cortex and medulla vary from 29-31 mm. and the cortex alone from 11-12 mm. in thickness (chart I).

In the work reported here, the number of renal corpuscles in the bovine kidney was determined using the technique described by Moore[3]. The number varied from 800,000 to 1,700,000 with an approximate average of 1,000,000 per kidney. Putter in 1911 (quoted in an article by Vintrup)[4] estimated the number of renal corpuscles in the ox at 4,025,000 per kidney which was much larger than our figures.

The number of renal corpuscles per field (1.5 mm.) varies from 5 to 23 depending on the age of the animal (chart II). There is a postnatal enlargement of the corpuscle from 78 microns at birth to 221 microns in diameter at six years of age. The Malpighian corpuscles consist of a capsule of Bowman and the glomerulus. Its capsule is lined by simple squamous epithelium. The afferent arteriole enters at the vascular pole and breaks up into a large number of capillaries. The blood leaves the glomerulus at the vascular pole through the efferent arteriole. The efferent is distinguished from the afferent by being smaller in size and has no internal elastic membrane. The average size of the afferent vessel is 24 microns with a variation of 15 to 36 microns. The efferent vessel averages 14 microns with a variation of 12 to 18 microns. The glomerulus in many instances does not completely fill Bowman's capsule. In a cross section of a single loop of the glomerulus, proceeding from the outside inward, the following

CHART I—Comparative Depths of the Cortex and Medulla According to Age

AGE	CORTEX AND MEDULLA	CORTEX
Fetus, 9 months	14-19 mm	3- 6 mm
11 months	20-25 mm	8-10 mm
11 months	25-29 mm	7-10 mm
11 months	30-35 mm	10-11 mm
12 months	24-29 mm	7-12 mm
12 months	22-30 mm	9-12 mm
12 months	19-23 mm	6- 9 mm
2 years	25-33 mm	8-10 mm
2 years	21-28 mm	9-12 mm
2 years	25-31 mm	9-11 mm
7 years	24-32 mm	9-12 mm
9 years	29-31 mm	11-12 mm

*Journal article No. 581, n.s., of the Michigan Agricultural Experiment Station. contribution from the Animal Pathology Section.

[1]Langham, R. F. and Hallman, E. T.: The bovine kidney in health and disease. J.A.V.M.A., xcv (1939) pp. 22-32.

[2]Sisson, Septimus: The anatomy of the domestic animals (2nd Ed.), W. B. Saunders Co., Philadelphia, (1914) pp. 564-566.

[3]Moore, A. A.: The total number of glomeruli in the normal human kidney. Anat. Rec., xlviii (1931) pp. 153-168.

[4]Vintrup, B. J.: On the number, shape and structure and surface area of the glomeruli in the kidneys of man and animal. Am. Jour. of Anat. xli (1928) pp. 123-151.

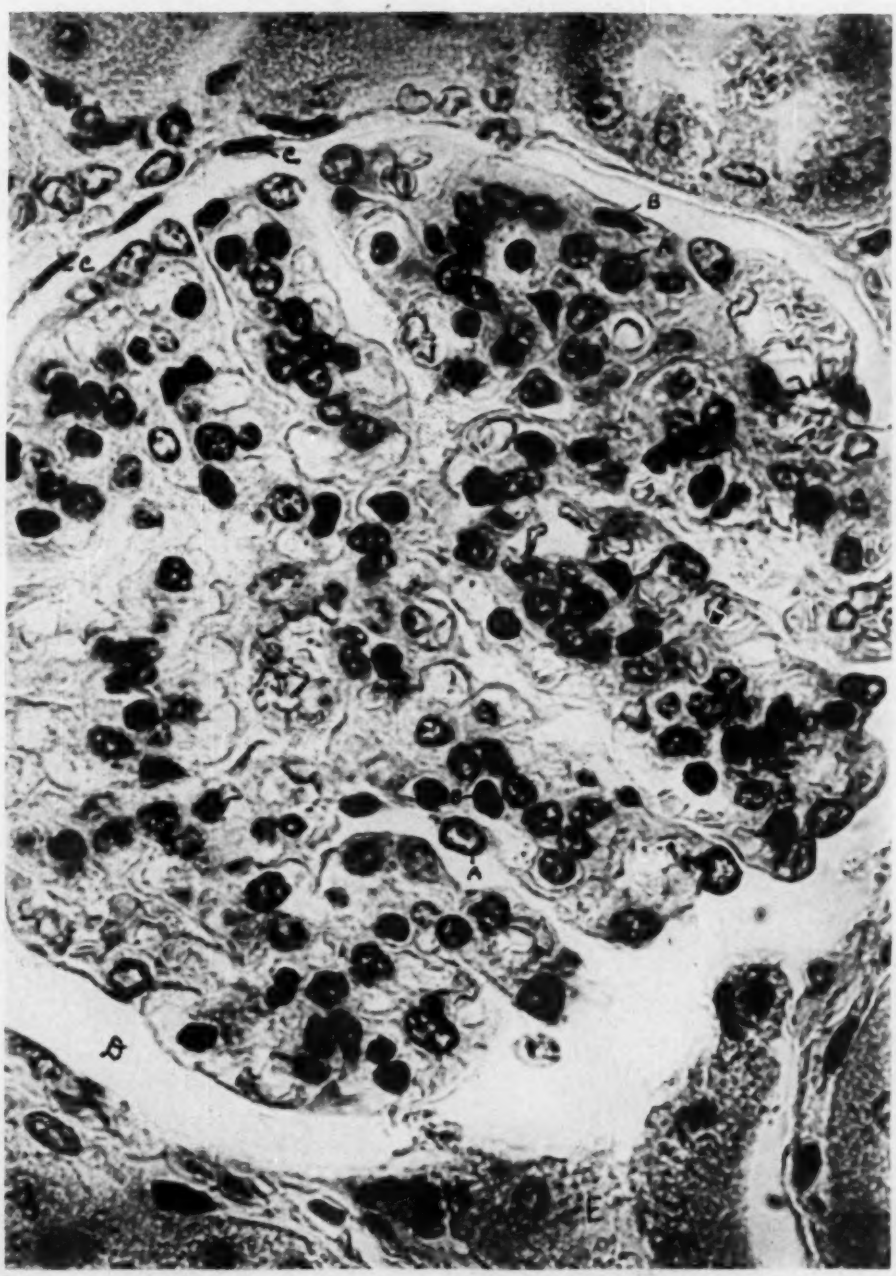

Fig. 1—Renal corpuscle. A. Visceral glomerular epithelial cell. B. Endothelial cell. C. Parietal epithelium of Bowman's capsule. D. Subcapsular space. E. Neck of proximal convoluted tubule. (X870).

Fig. 2—Glomerulus and neck of proximal convoluted tubule. A. Glomerulus. B. Neck of proximal convoluted tubule. C. Brush border. (X830).

CHART II—Number of Renal Corpuscles and Ratio of Endothelial to Visceral Epithelial Cells

		N*	R**
Full Term Fetus	Max.	32	1 : 8.5
	Min.	10	1 : 2.4
	Av.	23	1 : 4.44
6 weeks	Max.	20	1 : 5.2
	Min.	10	1 : 1.4
	Av.	14	1 : 2.75
3 months	Max.	13	1 : 3.7
	Min.	4	1 : 1.7
	Av.	8	1 : 2.54
11 months	Max.	8	1 : 4.0
	Min.	2	1 : 1.6
	Av.	5	1 : 2.52
1 year	Max.	10	1 : 2.8
	Min.	6	1 : 1.1
	Av.	8	1 : 1.8
1 year	Max.	8	1 : 2.5
	Min.	3	1 : 1.6
	Av.	5	1 : 1.95
2 years	Max.	8	1 : 3.2
	Min.	2	1 : 1.5
	Av.	5	1 : 1.95
2 years	Max.	15	1 : 2.5
	Min.	6	1 : 1.0
	Av.	10	1 : 1.54
2 years	Max.	8	1 : 3.0
	Min.	4	1 : 1.38
	Av.	6	1 : 2.07
4 years	Max.	8	1 : 1.8
	Min.	3	1 : 1.0
	Av.	5	1 : 1.25

The average for each animal represents counts on 25 glomeruli.

*Number of renal corpuscles in miscroscopic field using a 10X ocular and a 16 mm objective.

**Ratio of endothelial cells to epithelial cells in glomerulus.

structures are recognized: visceral epithelium, basement membrane, reticular fibers (potential tissue space), and vascular endothelium. The viseral epithelial cell nuclei are larger and more spherical than the endothelial cell nuclei and have a nucleoli. The nuclei average 7 to 8 microns in diameter. The endothelial cells are more elongated, contain more chromatin, and stain darker (fig. 1). The nuclei measure from 3 to 4 microns in diameter.

By the use of a special stain (Heidenhain's aniline blue) the number of visceral epithelial cells and endothelial cells were counted and the ratio was approximately two epithelial cells to one endothelial cell (chart II).

The short neck of the proximal convoluted tubule of the bovine is different from the human in having a definite brush border, and the cells are higher (fig. 2). The average diameters of the proximal convoluted tubules show a variation from 38 microns at one month of age to 57 microns at six years. The epithelium has been described as truncated pyramidal cells[5] with a definite brush border (fig. 3). The nuclei are spherical and contain fine chromatin particles. The cytoplasm is abundant and quite granular, although it does not stain as deeply with eosin as the cytoplasm of distal convoluted tubules. The average diameters of the straight terminal portion of the proximal convoluted tubules show a variation of 23 microns at one month, to 41 microns at six years. They still retain the typical brush border (fig. 5).

The average diameters of the thin limb of Henle's loop show a variation from 12 microns at one month to 25 microns at six years. These limbs have a thin squamous-like epithelium with a pale staining cytoplasm. Because of the relatively small amount of cytoplasm, the spherical nucleus bulges into lumen. The nucleus has fine chromatin particles and a nucleolus can be seen in many of the cells. Due to the thinness of the walls it is sometimes difficult to differentiate the thin limbs from blood capillaries (fig. 6).

The average diameters of the thick limb of Henle's loop show a variation from 17 microns at one month to 36 microns at six years. The epithelium varies somewhat in the thick limb. In many cases it is low cuboidal, but in other places it is almost simple squamous. The cytoplasm is more abundant than that found in the cells of the thin limb and stains deeper with eosin. The oval nuclei contain an abundance of chromatin which gives them a dark appearance (fig. 4). As the ascending limb returns to

[5]Maximow, A. A., and Bloom, W.: A textbook of histology. (2nd Ed.) W. B. Saunders Co., Philadelphia, (1934) p. 472.

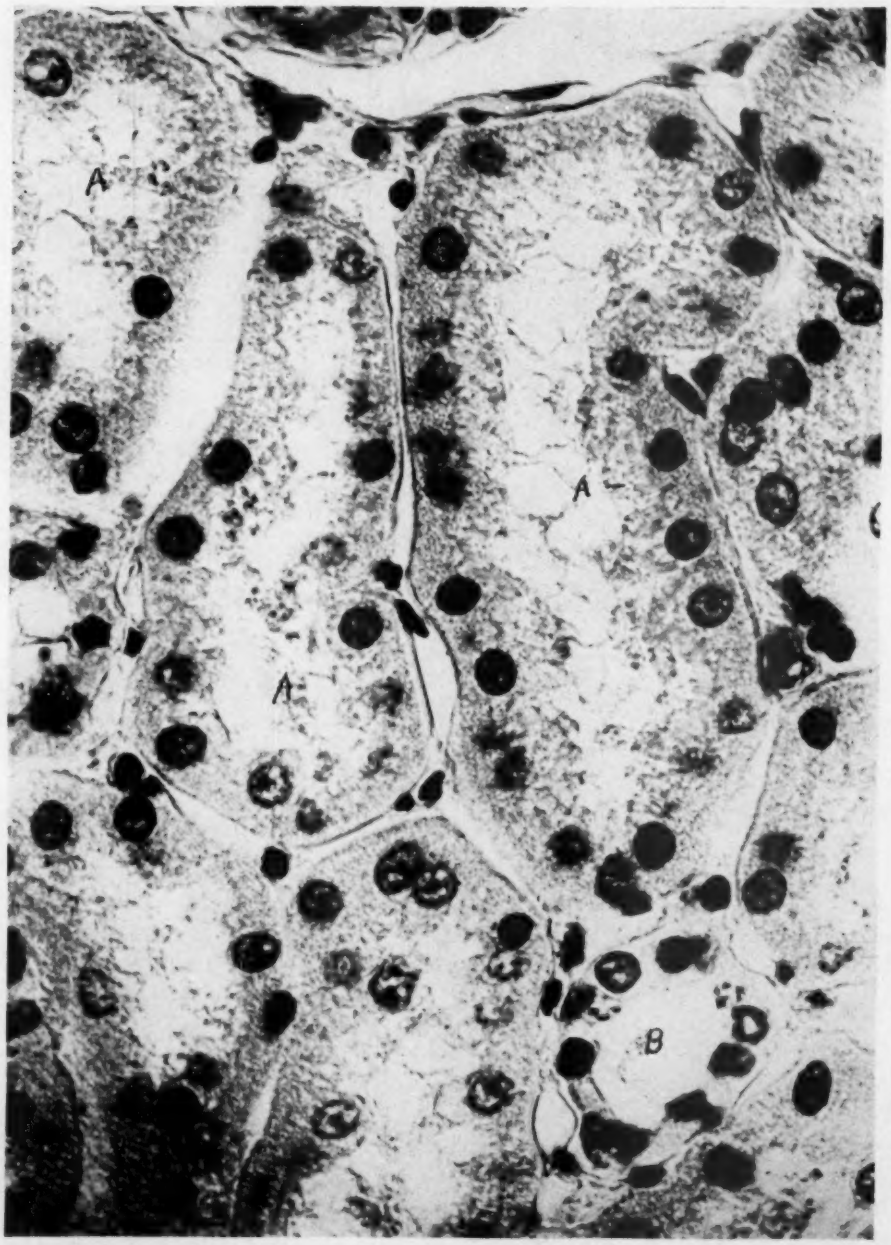

Fig. 3—A. Several proximal convoluted tubules to show truncated pyramidal cells and their brush border. B. Ascending arm of Henle's loop. (X823).

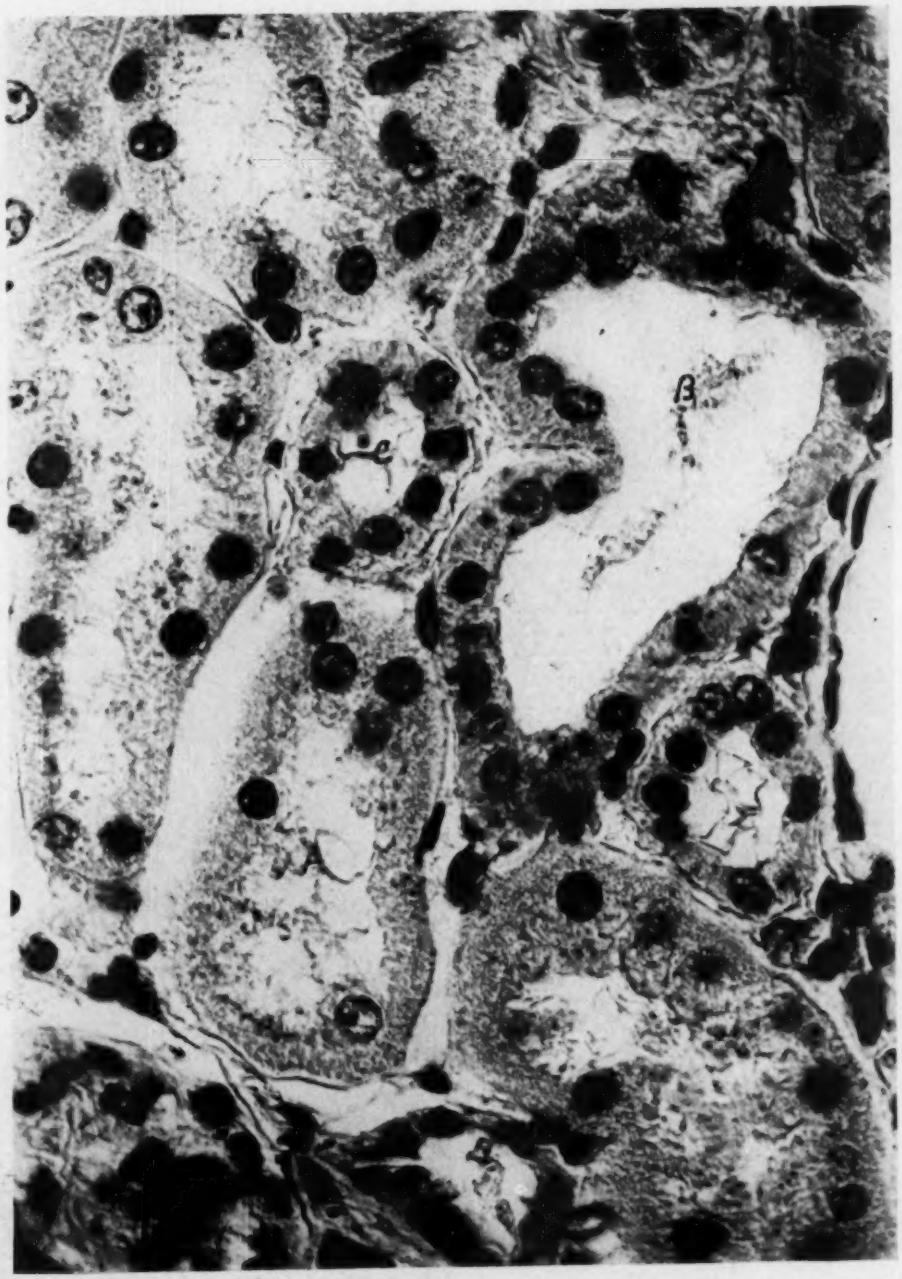

Fig. 4—Labyrinth of cortex. A. Proximal convoluted tubule. B. Distal convoluted tubule. C. Ascending arm of Henle's loop. (X875).

Fig. 5—Outer zone of the outer medulle. A. Terminal portion of proximal convoluted tubule. B. Ascending limb of Henle's loop. C. Collecting tubule. (X870). The retraction of the epithelium from the basement membrane is an artefact.

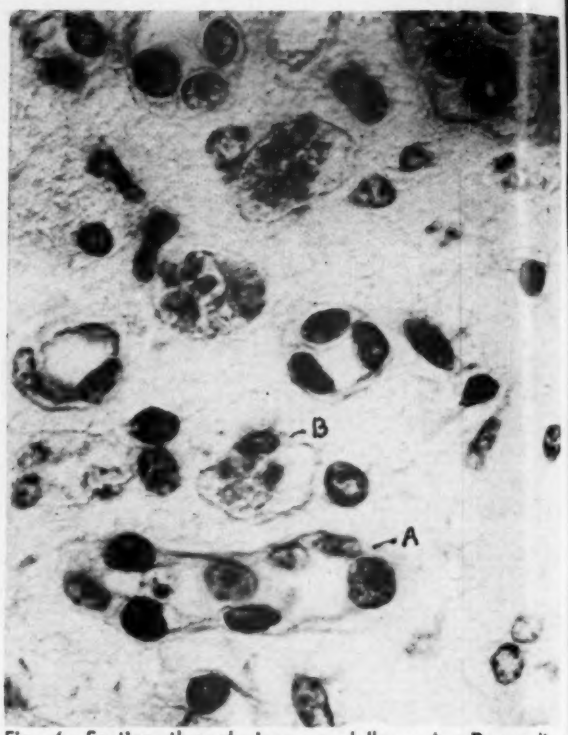

Fig. 6—Section through inner medullar. A. Descending limb of Henle's loop. B. Blood capillary. (X769).

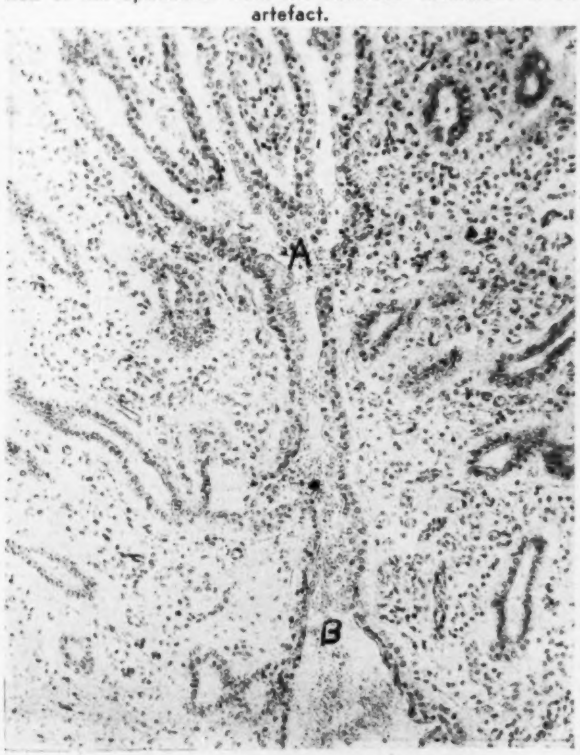

Fig. 7 — Section through inner medulla. A. Collecting tubules fusing to form duct of Bellini. B. Duct of Bellini. (X160).

Fig. 8—Cuboidal epithelium of collecting tubule. (X1030).

its renal corpuscle and attaches to the vascular pole, the epithelium becomes more cuboidal, the nucleus is more spherical, they are more numerous, and each contains a large central mass of chromatin.

The average diameters of the distal convoluted tubules show a variation from 36 microns at one month to 46 microns at six years. In comparing the distal convoluted tubules with the proximal convoluted tubules, several distinctive features can be seen. The distals have a cuboidal epithelium with no brush border (fig. 3). The cells are smaller; therefore the nuclei are more numerous and stain darker due to the greater abundance of chromatin. The cytoplasm is not so abundant and less granular. It takes a deeper stain with cosin. The length of the distal convoluted tubule is relatively short in comparison with that of the proximal convoluted tubules.

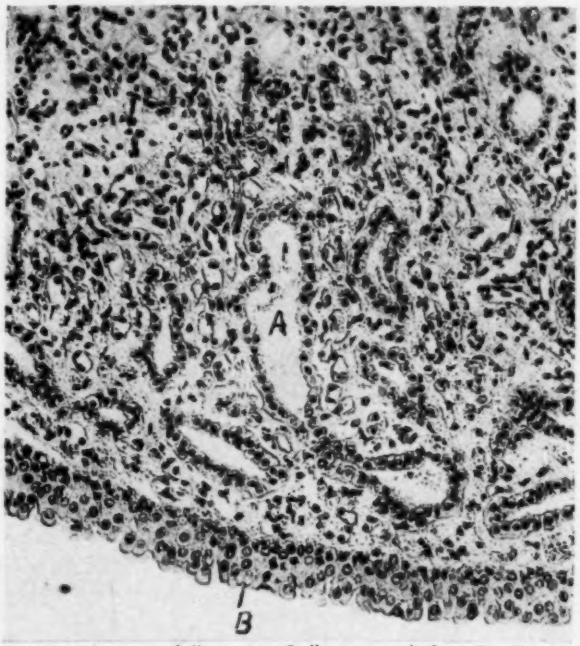

Fig. 9—Inner medulla. A. Collecting tubule. B. Transitional epithelium of papilla. (X160).

The straight collecting tubules are joined by connecting tubules all through the cortex, but in the outer medulla no fusions take place. However, in the inner medulla the tubules join at acute angles with similar tubules at least four or five times (fig. 7). By making several fusions these tubules form the ducts of Bellini which open at the area ribrosa at the apex of the papilla (fig. 7). The epithelium of these tubules is cuboidal (fig. 8). The nuclei are round and take a central position in the cells. The chromatin is quite abundant. The cytoplasm stains lightly with eosin. The average diameters of the straight collecting tubules in the cortex and outer medulla show a variation from 27 microns at one month to 37 microns at six years.

In the ducts of Bellini, the epithelium acquires a tall columnar form. The cells retain the other characteristics of those of the collecting tubules. The surface of the papilla is covered by a transitional epithelium (fig. 9).

Studies on Sheep, with the Demonstration of Pasteurella Localized in the Central Nervous System*

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DURING THE COURSE of investigations on central nervous infections, specimens from a flock of sheep in which losses occurred were referred to us as suspected cases of listerellosis.

The flock consisted of feeder lambs together with native and western ewes. Losses were occurring in both groups of ewes before and after lambing. A large part of the lamb crop was lost. In some cases, the course of the disease ran a week or more before terminating fatally, but in other instances death occurred a day or two following the appearance of the first clinical symptoms.

CLINICAL PICTURE

Some of the sheep showed opisthotonos and spasmodic motions of the fore legs. In some, the cornea was milky in appearance and there was considerable conjunctival exudate. The temperatures of the sheep submitted to the laboratory were subnormal (95 to 100 F.). These were in the late stages of the disease. The ewes showed numerous Oesophagostomum nodules, but very few nematodes were present in the abomasum and small intestine. One ewe showed a slight subcutaneous edema over the ventral thoracic region. The carcasses carried considerable fat. Postmortem examination revealed no significant gross changes. The liver appeared friable and the heart muscle was slightly degenerated. The brain and meninges were slightly congested. The surface of the meninges appeared dull and more or less translucent.

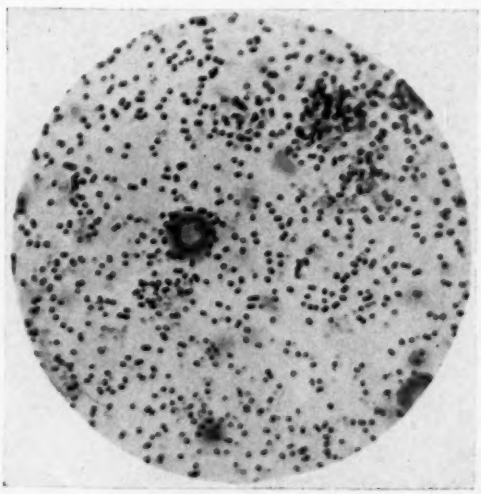

Fig. 1—Pasteurella organisms—smear from agar slant. (X1125.)

HISTOPATHOLOGY

Frozen sections of the brain from various field cases revealed early degenerative changes and cellular increase. Numerous polymorphonuclear and monocytic cells were found in the blood vessels as well as in the surrounding tissues. Rarely, small accumulations of these cells were found. The meninges were similarly affected. Extensive foci of infiltration and perivascular cuffing as are found in listerellosis were not present in any of the sheep examined in this flock. The gross and microscopic changes were not specifically diagnostic.

FIELD CASES

1) This subject was a ewe. Cultures made from the brain were negative. Triturated brain tissue suspended in sterile saline (0.2 cc.) was inoculated intracranially into a rabbit. About 60 hours later, the rabbit was destroyed while in a moribund condition. A pure culture of Pasteurella was recovered from the brain of the rabbit (fig. 1). In stained smears from agar slants, the morphology of the organism simulated that of a diplococcus, whereas in smears of blood or exudates from experimentally infected animals

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the organism presented the characteristic bipolar appearance of the Pasteurella group.

2) The second subject from this flock was studied in the same manner. A rabbit inoculated intracranially with brain tissue died 24 hours later and a Pasteurella organism was recovered from the brain. A second rabbit was likewise inoculated 19 days later with some of the original sheep brain stored in 50 per cent glycerin. This rabbit appeared dull and on the sixth day it rolled its head and swayed from side to side. When placed on its side it showed difficulty in getting up. It was killed on the ninth day. Cultures of the brain, spleen, liver, and heart blood were negative. At this time, the third rabbit and one guinea pig were inoculated intraperitoneally with an emulsion made from the original sheep brain. One guinea pig received an intracranial injection of the same material. The infectious agent did not remain viable in the glycerin-stored brain tissue since none of the latter series manifested clinical symptoms.

3) A lamb included with the two previously described cases died the day before these were referred to us for study. The head had been removed from the body and held under refrigeration until the next day. This material was not cultured or inoculated, but histologic sections of the brain were examined. The cerebellar meninges showed considerable inflammatory reaction with some cellular infiltration similar to the first two cases (fig. 2). Perivascular and extensive focal infiltration were absent.

4) Specimen 4, a lamb, was included with cases 1 to 3. Brain tissue was stored in 50 per cent glycerin under refrigeration, pending the outcome of histologic studies and animal inoculations of the other material.

Intracranial inoculation of brain tissue from this case failed to kill the rabbit. The microscopic changes were similar to those of the previous cases. The changes did not resemble those associated with listerellosis and certain virus infections.

5) The brain of a dead ewe from this flock presented the same microscopic pathology as that described above. After storage in glycerin for 16 days, this material failed to cause death in laboratory animals upon intracranial inoculation.

6) A live lamb from this flock was held for observation. It was unable to get up and was destroyed by bleeding. The symptoms manifested were those of a central nervous involvement. Microscopic examination of the brain revealed only a slight cellular increase, consisting chiefly of polymorphonuclear and monocytic cells together with slight degenerative changes. Perivascular cuffing was absent. Cultures of the brain, liver, spleen, and heart blood were negative.

A rabbit inoculated intracranially with 0.2 cc. of brain emulsion from this case died within 24 hours. Pasteurella was recovered from the heart blood, liver, and brain of the rabbit. Microscopic study of the rabbit brain revealed a marked leucocytosis involving large areas. The tissues around the lateral ventricles were severely involved. The meninges were markedly infiltrated with polymorphonuclear cells. Some fields showed necrosis and suppuration.

A second rabbit was injected with brain tissue from this lamb after the tissue had been stored in 50 per cent glycerin for four days. The rabbit succumbed in less than 24 hours. Pasteurella was recovered from the rabbit. Frozen sections showed a pathologic picture similar to that in the first rabbit.

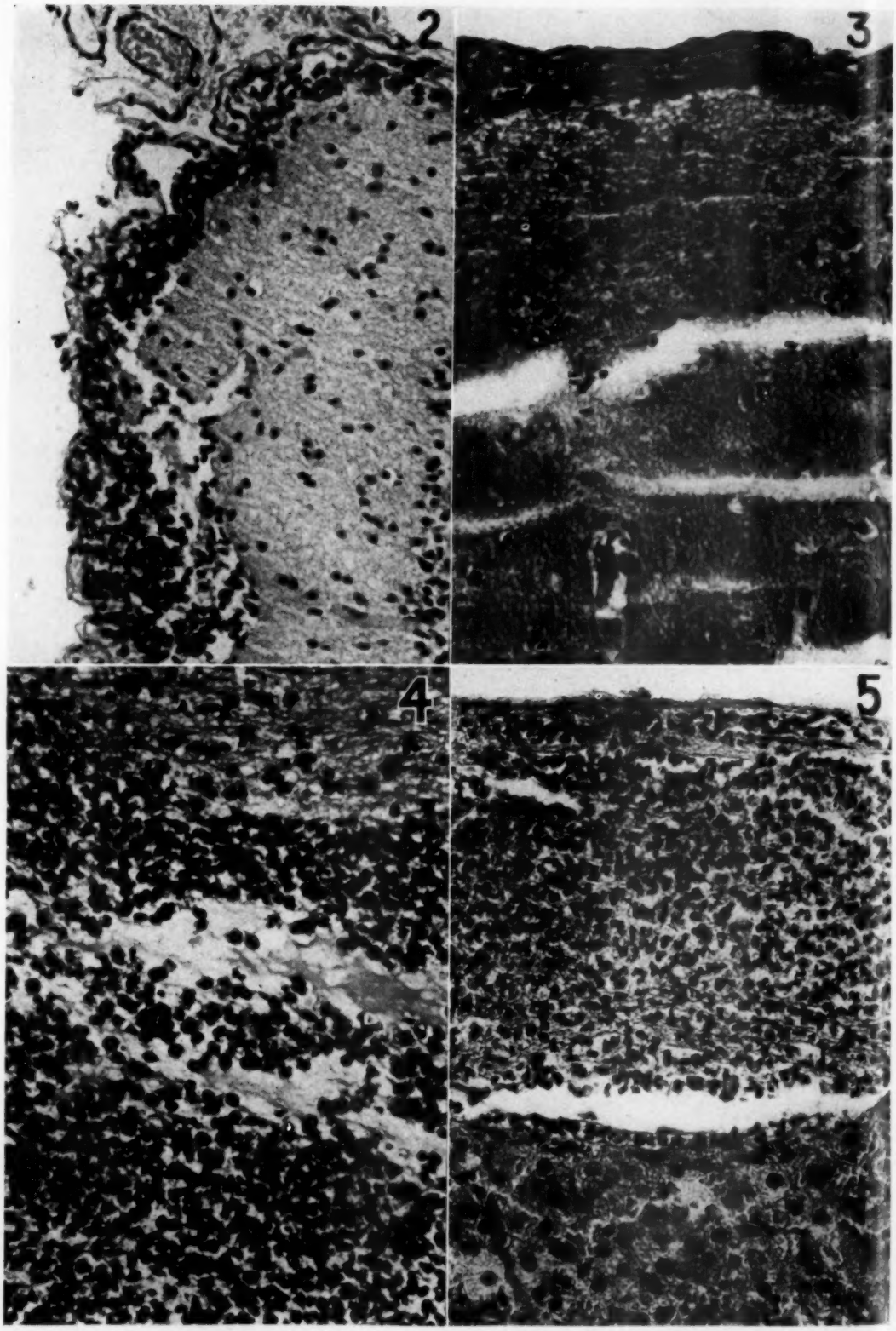
A guinea pig receiving 0.1 cc. of the same suspension of lamb brain intracranially likewise died within 24 hours and a pure culture of Pasteurella was recovered.

This culture was passed by intracranial and intraperitoneal inoculations through long series of rabbits and guinea pigs with the same results. Death occurred in the intracranially injected animals in less than 24 hours. Intraperitoneal inoculations resulted in death in from less than 24 hours to 96 hours or more, depending upon the age of the cultures.

Because of the divergent views as to the status of Pasteurella in its relation to the primary cause of losses, we were reluctant to assign to it the sole cause of the losses in this flock.

7) Another subject from this flock was destroyed by bleeding. Cultures of the brain, spleen, heart blood, and liver were negative. Brain tissue, liver, spleen, and clotted heart blood were triturated in separate sterile mortars and suspended in saline solution. These four suspensions were inoculated intraperitoneally into separate groups of guinea pigs. The three guinea pigs which received the suspension of sheep brain died within 24 hours and Pasteurella was recovered. The guinea pigs inoculated with liver, spleen, and heart-blood suspensions remained well, manifesting no clinical symptoms.

8) In order to confirm the results encountered in the studies on field case 7, the farm was visited and another sick lamb was obtained for further study. The clinical picture resembled that often seen in ovine listerellosis. Mucus was discharged from the nostrils, but pneumonia was not found upon autopsy. The cornea had a milky appearance. The lamb showed early posterior incoördination. The same investigational procedure carried out in case 7 was followed in the study of this animal. Cultures of organs by the inoculation of broth and agar slants were negative. A few isolated colonies appeared on agar slants inoculated with the triturated brain tissue of this sheep. These proved to be colonies of Pasteurella organisms.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Two guinea pigs were inoculated intraperitoneally with suspensions of brain tissue of lamb 8. The inoculations were made at about 5 p. m. on May 6 and both guinea pigs were found dead on the morning of May 8. Pasteurella organisms were recovered from the spleen, liver, and heart blood of one guinea pig, but the cultures made from the brain were negative. From the second guinea pig, Pasteurella organisms in pure culture were obtained from the brain, spleen, liver, and heart blood. Separate suspensions of spleen, liver, and heart blood of this lamb caused no reactions in other groups of guinea pigs inoculated intraperitoneally.

BACTERIOLOGY

The organisms isolated were gram-negative, short, plump rods. In stained smears from agar slants inoculated from fatal cases, the morphology of the organism simulated that of a diplococcus, whereas in smears of blood or exudates from experimentally infected animals the organism presented the characteristic bipolar appearance of the Pasteurella group. In serum broth, the organisms produced a uniform turbidity and they were arranged in pairs and as single cells. There was considerable variation in the shape of the cells. Some were very short, appearing as small cocci. The majority, however, were short, plump rods. The organisms were nonmotile, nonhemolytic, and produced no change in litmus milk. A small amount of H_2S was produced and gelatin was not liquefied. The following carbohydrates were fermented with the production of acid, but no gas; dextrose, sucrose, galactose, mannose, xylose, and mannite. The organism failed to attack lactose, trehalose, arabinose, inositol, dulcitol, raffinose, maltose and salicin. The above characteristics are identical to those of *Pasteurella multocida* group II as classified by Rosenbusch and Merchant.[1]

[1]Rosenbusch, C., and Merchant, I. A.: A study of the hemorrhagic septicemia Pasteurellae. J. Bact., xxxvii (1939), pp. 69-89.

CAPTIONS FOR ILLUSTRATION ON OPPOSITE PAGE

Fig. 2—Meningitis in sheep. Field case. (X300.)
Fig. 3—Meningitis and submeningeal reaction in experimentally infected rabbit. (X300.)
Fig. 4—Severe leucocytic infiltration around lateral ventricles in experimentally infected rabbit. (X300.)
Fig. 5—Exudate on capsule of liver of guinea pig inoculated intraperitoneally. Course of disease 2 days. (X300.)

EXPERIMENTAL INOCULATION OF RABBITS AND GUINEA PIGS

Rabbits and guinea pigs which received intracranial inoculations of young, pure cultures of the strain of Pasteurella recovered from this epizoötic succumbed in 24 hours or less. Microscopic study of the brains revealed meningitis with severe leucocytic infiltration (fig. 3). The tissues surrounding the lateral ventricles presented necrosis and extensive leucocytosis (fig. 4).

Rabbits and guinea pigs which received intraperitoneal injections of this strain of Pasteurella died in from 24 hours to 4 days or more. Cultures held at room temperature for six or more days before injection produced a longer course of the disease which was accompanied by emaciation and rough coat. A heavy, yellowish-colored exudate appeared on the peritoneal linings of the abdomen with extensive adhesions in the cases of long duration. In the latter type, pneumonia and pericarditis also appeared.

In the cases of short duration, the tunica serosa of the liver was destroyed and the capsule of Glisson was covered with leucocytes enmeshed with fibrin (fig. 5). In the cases of longer standing, this material had undergone organization with considerable connective tissue proliferation, causing adhesions (fig. 6).

EXPERIMENTAL INFECTION OF SHEEP

Sheep A received 9 cc. of a very dilute suspension of organisms in saline solution intravenously. The sheep was found dead the next morning. Pasteurella was recovered from the brain, heart blood, spleen, and liver. The brain showed a general infiltration consisting chiefly of leucocytes, but perivascular cuffing was absent. The changes simulated those found in field cases.

Pregnant ewe B received 5 cc. of dilute saline suspension of Pasteurella organisms subcutaneously on May 27. The following two days the animal appeared sluggish and showed daily temperatures of 106.4 and 104.8, following which it dropped to normal. On June 5, five cc. of a saline suspension of the same strain of Pasteurella was inoculated intravenously. This second inoculation caused no visible clinical reactions or marked rise in temperature.

The ewe was sacrificed for autopsy on June 13. The only significant changes that were found consisted of slight congestion and friability of the liver. The kidneys appeared grayish brown and contained numerous retention cysts. The splenic nodules were enlarged. Numerous Oesophagostomum lesions were present in the intestine. Cultures of the brain, liver, heart blood, kidneys, and spleen were negative.

Microscopic examination of the brain revealed considerable degenerative changes in the vessels and some hemorrhages. An increase of leucocytes appeared throughout all sections. A few areas of early necrosis and leucocytes were found.

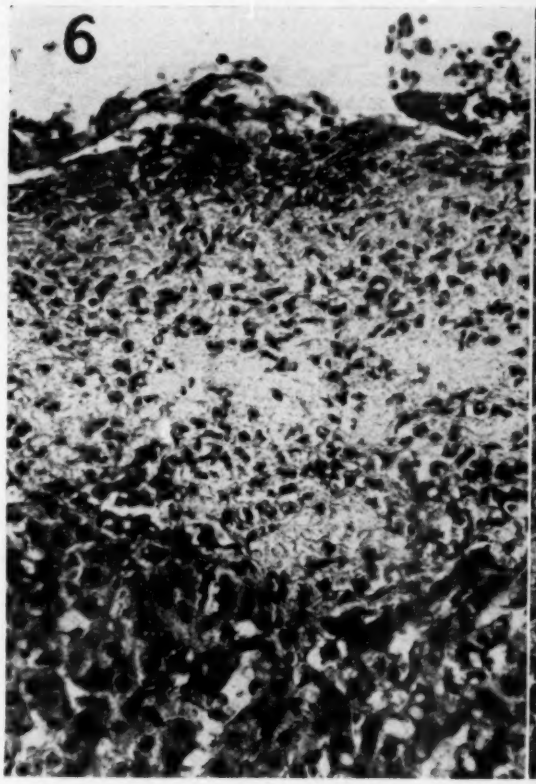

Fig. 6 — Organization of exudate on liver of guinea pig which died 4 days following intraperitoneal injection of culture. (X300.)

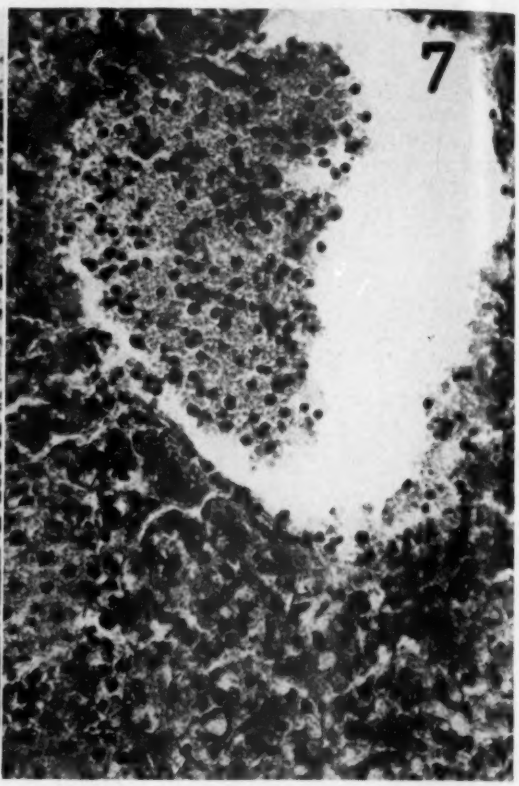

Fig. 7—Epithelial degeneration and leucocytic infiltration of liver of sheep experimentally infected. Blood vessels contain many leucocytes. (X300.)

Sheep C received 5 cc. of Pasteurella suspension intravenously on May 27 and died June 3. No fluid was found in either pleural or abdominal cavities. Pneumonia was present. The spleen pulp was increased in amount and appeared like jam in consistency. The heart showed no hemorrhages, but the myocardium appeared slightly swollen and gray. The kidneys and gastrointestinal tract showed no significant gross changes. A few isolated colonies of Pasteurella appeared on slant cultures made from the brain, whereas cultures from the liver, spleen, heart blood, and kidneys exhibited numerous colonies.

The microscopic changes in the brain were pronounced, including hemorrhages and degenerative changes. Many of the vessels contained large numbers of leucocytes.

Lamb D received 5 cc. of the same culture intraperitoneally and died 24 hours later. The heart showed a few petechiae. The serosa of the abdomen also was the site of petechiation. The liver was congested and friable. The spleen was slightly enlarged and the pulp appeared soft. Pasteurella in pure culture was recovered from the brain, liver, heart blood, spleen, and kidneys. Microscopic examination of the brain revealed a few hemorrhages and extensive leucocytosis.

Lamb E received 5 cc. of culture intravenously and died within 24 hours. A few hemorrhages were found on the serosa of the spleen and abomasum. The heart showed only a few petechiae on the epicardium. No fluid was found in the abdominal cavity. Pasteurella was recovered from the brain, liver, heart blood, and spleen.

Lamb F received 5 cc. of Pasteurella suspension intravenously and died within 48 hours. The bacteriologic and pathologic findings were similar to those in the case of lamb E, except that in this case the heart showed more extensive petechiation.

Ewe G received 5 cc. of saline suspension of the same Pasteurella strain intravenously and died within 24 hours. The meninges were somewhat roughened and the brain appeared slightly softened. Microscopic study revealed extensive leucocytic infiltration and degenerative changes. Gross hemorrhages were not observed on the serous membranes. The liver showed foci of necrosis and leucocytic infiltration. The blood vessels contained numerous leucocytes (fig. 7). Pasteurella organisms were recovered from the brain, liver, heart blood, and kidneys.

Lamb H received 5 cc. of saline suspension of Pasteurella organisms intraperitoneally. There was no significant rise in temperature and the animal appeared in good condition seven days later, when it was sacrificed for postmortem study. Cultures of the brain, liver, heart blood, kidney, and spleen were negative. The brain showed the same type of degenerative changes and leucocytic infiltration as other cases of the series.

EXPERIMENTAL INOCULATION OF SWINE

Pig A received 5 cc. of a dilute saline suspension intraperitoneally of the strain of Pasteurella recovered from this epizoötic. The pig showed no significant rise in temperature or untoward clincal manifestations. It was killed eight days after the inoculation. Cultures of the brain, liver, gall bladder, heart blood, spleen, and lungs were negative. No significant changes were found at autopsy.

Pig B, inoculated intraperitoneally with similar material, likewise proved negative on bacteriologic examination when destroyed eight days following the inoculation.

DISCUSSION

When the first cases were submitted, the owner reported that the ewes were being fed sorghum silage, soybean hay, and oats. The feeder lambs received sorghum silage, ground bean hay, and ground cob meal. After the cloudy appearance of the cornea in some of the sheep was noted and the feed examined, it was suggested that yellow corn be included in the ration and that as soon as pasture became available the animals be moved from the dry lots. There was considerable improvement after the sheep were placed on pasture.

In view of the divergent views held regarding the rôle of Pasteurella as a primary factor in the cause of disease in some species, and as a result of our findings in this epizoötic among sheep, we feel that the losses were in part associated with one or more nutritional factors.

The pathology of the brain was strikingly different from that found in listerellosis. The microscopic changes found in experimental subjects inoculated with the strain of Pasteurella organisms from this epizoötic resembled those of the field cases, except that in the intracranially injected cases the lesions in the brain were more severe and included necrobiotic changes and dense masses of leucocytes.

These studies, especially on the last two field cases, offer additional support of the citeria for diagnosis as recommended by us in listerellosis and other central nervous disorders, that is, histopathologic examination of the brain tissue and intracranial inoculation of suspensions of brain tissue into laboratory animals, even though the inoculation of field material on bacteriologic mediums proves negative.

CONCLUSIONS

Pasteurella organisms were recovered during an epizoötic among sheep which manifested clinical symptoms characteristic of a central nervous disturbance. The organisms were recovered from the brain of experimentally inoculated animals and in one case by direct inoculation of a bacteriologic medium with a suspension of infectious brain material.

One or more nutritional factors were believed to be contributory in the course of the disease.

The pathology of the brain was distinct from that associated with ovine listerellosis.

The Pasteurella organisms recovered from this flock were identical to those of *Pasteurella multocida* group II as classified by Rosenbusch and Merchant.

Homogenized Sulfanilamide-In-Oil Intramammary Injections* In Bovine Mastitis*

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Newark, Delaware

DURING THE PAST decade, research workers on animal diseases have devoted much time to the study of bovine mastitis. The field has produced considerable literature on many phases of the problem. In this report, no attempt is made to review the literature that does not have a direct bearing upon the investigation.

Various drugs have been used to destroy infectious agents in the udder of cows, but until recently, they brought but little success. Since the discovery of sulfanilamide and its therapeutic powers against streptococci in human infections, many investigators have used this drug in attempts to cure bovine mastitis. Allott[1] reported improvement of three cows suffering from mastitis by the oral administration of sulfanilamide. However, owing to insufficient laboratory data on the milk samples, no conclusion on the value of sulfanilamide in these cases is warranted. Bauer and Gunderson,[2] Johnson and Miller,[3] Gildow, Fourt and Shaw,[4] reported that oral administration of sulfanilamide, even in maximum doses, did not eliminate the mastitis streptococci from the infected udders. Other authors, including Hoge, Halversen and Cherrington,[6] Miller, Mingle, Murdock, and Heishman,[6, 7] Stevenson,[8] Klein, Kleckner and Parry,[9] Bryan and Arnold,[10] Leopold,[11] King, Reid, Hare, Greenfield, and Davies,[12] Veenbaas and Sjollema,[13] Heishman and Miller,[14] and Foltz,[15] and McEwen, Pizer and Paterson,[16] who have attempted to cure bovine mastitis by administering sulfanilamide orally, have shown that the drug is ineffective in eliminating streptococci permanently from the infected udder. Even in cases where the doses were large enough to produce toxic effects the infection persisted.

Little[17] reported that the oral administration of as much as 1,361 Gm. of sulfanilamide per cow failed to destroy the streptococci in the injected quarters. In this laboratory, 950 Gm. of sulfanilamide administered orally over a period of five days to a cow infected with *Streptococcus agalactiae* was sufficient to maintain a blood and milk level of over 20 mg. per cent, but failed to destroy the infection within the udder.

STUDIES *in vitro* (LANCEFIELD GROUP B)

Sulfanilamide has been found to be effective against infections caused by hemolytic streptococci of the Lancefield Group A. Against the Group B streptococci, the results reported are by no means so favorable. In order for sulfanilamide to be effective against streptococcic infections, it is necessary to maintain not only an effective concentration of the drug for some time, but also an increase of from four to five degrees above the normal body temperature, as shown by White and Parker[18] on Lancefield Group A streptococci. Based on this hypothesis, the failure of sulfanilamide to destroy streptococcic infections in the udder of cows when the drug was administered orally may be due to insufficient concentration of the drug in the tissues, even when the dosage had reached maximum tolerance at normal body temperature.

The following experiments were carried out with the view of determining the relationship of concentration and temperature to the bactericidal properties of sulfanilamide and to test the correctness of the above postulate.

In the first experiment, the concentration was maintained at 20 mg. per cent, since experience has shown that this concentration of sulfanilamide in the blood is

*From Haskell Animal Disease Research, University of Delaware.

very close to the limit of tolerance. The temperature employed was 37 C., which is approximately, or slightly below, the normal. The streptococci employed in these *in vitro* tests were strains of *Str. agalactiae* (Lancefield Group B) recently isolated from infected cows. The study revealed that at body temperature (37 C.) sulfanilamide in a concentration of 20 mg. per cent has no bactericidal power against *Str. agalactiae.*

When the study was repeated with the culture tubes incubated at 40.5 C. instead of 37 C., the streptococci were killed after

CHART I—Effect of Sulfanilamide on Streptococcus Agalactiae *in vitro* Tests

Log of Bacterial Population per cc.
Incubation Time in Hours
— — — — 20 mg. per cent sulfanilamide at 37 C.
———— Control at 37 C.
— — — — 20 mg. per cent sulfanilamide at 40.5 C.
. . . . . Control at 40.5 C.

incubating 32 hours. By referring to chart I, it will be noted that at 40.5 C. the growth rate in the untreated or control tubes was at first retarded but later the bacterial population reached the normal maximum. In the treated tubes (sulfanilamide 20 mg. %), growth after a brief delay proceeded normally for a few hours followed by a rapid death rate which was complete at about 32 hours.

These experiments were repeated with a number of strains of *Str. agalactiae* with only slight variations in the results. It may be concluded, therefore, that while different strains of the organism vary in their resistance, there is a definite relationship between the temperature and the germicidal properties of sulfanilamide for Lancefield Group B streptococci. This observation is substantiated by an experiment reported by Miller *et al.*,[6] one of the few cases in which oral administration was followed by a complete cure. The cow was receiving sulfanilamide orally in amounts sufficient to maintain a blood concentration slightly over 20 mg. per cent for 48 hours. This animal for unexplained reasons had a rise in temperature to 104.4 F. (40+ C.) which was maintained for several days. These workers report that during the course of the treatment the streptococci were apparently eliminated from the udder.

As chronic streptococcic mastitis is usually a nonfebrile infection, the above observations explain the failure of sulfanilamide when administered orally. The above studies suggest, however, that if a practical method could be devised for inducing artificial fever in the cow, sulfanilamide administered orally would probably cure the majority of cases. Attempts made in this laboratory to produce artificial fever in the cow were not successful.

Experiments were next carried out to study the germicidal properties of sulfanilamide at 37 C., using various concentrations of the drug against a number of strains of *Str. agalactiae.* This study revealed that strains of *Str. agalactiae* show marked variation in their tolerance for sulfanilamide at normal body temperature. Some strains were destroyed in a concentration of 0.1 per cent, whereas others required a higher concentration, the most resistant requiring a concentration of 1.0 per cent. This variation in drug resistance possessed by different strains of the organism was demonstrated by a series of experiments wherein the cup test method was used. It is planned to report these studies later.

As a result of these *in vitro* tests, it was thought that similar bactericidal action might take place if sulfanilamide were in-

troduced into the infected udder in sufficient amounts to raise the concentration level in the infected tissues to such a point as to destroy the organisms. It was found that sulfanilamide can be readily introduced into the udder of a cow by properly suspending the drug in light, liquid petrolatum. When sulfanilamide is introduced directly into the udder *via* the teat duct, the drug is readily absorbed by the glandular tissue, and it is possible to establish and maintain a level of concentration sufficient to destroy the streptococci. This is shown in table 1. It will be seen that the animal received 40 cc. of sulfanilamide-in-oil in the right rear quarter on three successive days at approximately 24-hour intervals. Samples of milk taken from the animal after the injection showed that high concentration of sulfanilamide was maintained in the treated quarter for several hours. Although the drug appeared in the milk of the other

TABLE I—Sulfanilamide Determinations* in Milk and Blood of Lactating Cow After Intramammary Injection Into the Right Rear Quarter of Sulfanilamide Homogenized in Liquid Petrolatum

Date	Amount of Sulfanilamide-Oil Mixture Injected	Free Sulfanilamide in Milk—Mg. Per Cent from Four Quarters				Free Sulfanilamide in Blood—Mg. Per Cent***
		Right Rear (Treated)	Right Fore (Untreated)	Left Rear (Untreated)	Left Fore (Untreated)	
1/5/42: 8:30 a.m.**	40 cc.	0	0	0	0	0
9:30 a.m.		No Sample	0.75	0.75	0.75	4.0 rt. vein
11:00 a.m.		560.0	24.0	0.5	1.0	2.0 lt. vein
1:00 p.m.		280.0	3.0	14.0	6.0	4.0 lt. vein
4:00 p.m.		200.0	5.0	10.0	6.0	1.5 rt. vein
1/6/42: 8:00 a.m.**	40 cc.	25.0	16.0	11.0	12.0	1.5 lt. vein
1:00 p.m.		400.0	2.0	2.0	3.0	4.0 rt. vein
4:00 p.m.		60.0	4.0	28.0	3.5	2.0 lt. vein
1/7/42: 8:00 a.m.**	40 cc.	10.0	1.0	1.0	1.5	1.5 rt. vein
1:00 p.m.		Oily 8000.0	4.0	2.0	3.5	5.0 rt. vein
4.00 p.m.		1800.0	2.5	2.0	4.0	No Sample
1/8/42: 8:00 a.m.		2.0	2.5	1.75	3.75	3.0 lt. vein
4:00 p.m.		2.0	1.0	0.75	1.0	No Sample
1/9/42: 2:00 p.m.		1.0	Trace	Trace	0.25	0.25 rt. vein
1/10/42: 9:00 a.m.		0.5	0.75	0.5	0.1	Trace lt. vein
1/12/42: 9:00 a.m.		0.75	0.75	0.75	0.1	No Sample

* = The LaMotte micro equipment was used for sulfanilamide determinations throughout this investigation.

** = Milk samples were collected first and the sulfanilamide homogenized in liquid petrolatum was injected into the right rear quarter after the cow was completely milked.

*** = Blood taken from subcutaneous abdominal vein; rt. = right, lt. = left.

quarters, the concentration was not high enough to destroy the streptococci at normal temperature. It will be noted, furthermore, that the sulfanilamide also appeared in the blood stream. Thus, it is possible, by this method of administration, to infiltrate the infected tissues with sulfanilamide in sufficient concentration to destroy most of the strains of mastitis streptococci; yet the amount of the drug escaping into the general body tissues is far below the maximum tolerance of the animal.

PREPARATION AND DOSAGE OF HOMOGENIZED SULFANILAMIDE

Sulfanilamide, unless properly mixed with the liquid petrolatum, does not make a satisfactory suspension for intramammary injection. The drug soon settles and forms clumps which mechanically interfere with the administration. After considerable experimental study, the most satisfactory preparation was found to be a homogenized product prepared as follows:

To 900 cc. of light, liquid petrolatum one pound (453.6 Gm) of sulfanilamide is added and mixed thoroughly by means of a mechanical mixer, until a uniform suspension is produced. The mixture when passed through a homogenizer forms a thin, uniform, creamy suspension which remains stable. One cc. of this mixture contains 0.378 Gm. of sulfanilamide. It is, therefore, an approximately 38 per cent suspension of homogenized sulfanilamide in light, liquid petrolatum.

The dosage is dependent on the resistance of the strain of *Str. agalactiae* and on the size of the mammary gland. In practice, it is advisable to employ a dose of sufficient size to destroy the more resistant strains. This may be accomplished in an udder of average size by employing 40 cc. as the initial dose and repeating at 24-hour intervals for four days. This constitutes one course of treatment, and any quarters showing infection thereafter should be retreated immediately, using double the dose. The treatment may be repeated with safety as often as required. Cows with very large udders should receive larger initial doses. There were no contraindications. This fact is in marked contrast to other agents which have been recommended for intramammary use in mastitis. It has been employed in acute, chronic, and subclinical mastitis with satisfactory results. It is nonirritating to the udder tissue and produces no changes in the physical appearance or quantity of milk secreted. It may be employed in either dry or lactating animals. The treatment has been used with satisfactory results in cows which were on semi-official tests for milk production. In lactating cows, the treatment should be administered after milking.

METHOD OF DIAGNOSIS

In all cases, the method of diagnosis employed in this study was bacteriological combined with clinical examination of the udder. The milk samples brought to the laboratory were plated on tryptose blood-agar plates.

Medium for plating	
Bacto-tryptose	20.0 Gm.
Bacto-dextrose	2.0 Gm.
Na_2HPO_4	8.0 Gm.
NaCl	2.5 Gm.
Agar	15.0 Gm.
Distilled water.....	1.0 liter

Adjust the hydrogen-ion concentration of the medium to pH 7.4 and sterilize by heating in the autoclave at 15 pounds extra pressure for a period of 15 minutes. Ten per cent citrated ox-blood is added to this medium and then poured into Petri dishes.

Each plate was divided into quadrants and a loopful of milk from each quarter was streaked on each quadrant. The plates were then incubated at 37 C and were observed after 24 hours, and again after 48 hours. The hemolytic and nonhemolytic streptococci were isolated and identified by means of physiological reactions and serological tests using the Lancefield technique and the rapid plate agglutination method. The degree of infection was determined by the number of organisms growing on the surface of the blood-agar plates from a 4 mm. loop.

TREATMENT AND RESULTS

After the infected quarters were detected by the blood-plate method and the streptococci identified, each infected quarter was first milked out completely and a dose of 40 cc. of the homogenized sulfanilamide-in-oil mixture was injected by means of a hypodermic syringe and a teat tube. Only the infected quarters of each cow were treated. First, each animal received one course of treatment consisting of four doses. Doses were given at intervals of 24 hours. Just before the daily treatment was given, milk samples were collected from all quarters of each animal and bacteriological

examinations were conducted to detect the presence of mastitis streptococci. About one week after the last dose was given, milk samples were again collected and examined. In cases where the infection reappeared, the course of four daily treatments, indicated above, was repeated. If the infection persisted, the daily dose was increased to 80 cc. These larger doses were used in animals with large udders.

Preliminary tests to determine the efficacy of this treatment were conducted on two cases of chronic *Str. agalactiae* infection of long standing. For a period of about two years prior to the present treatment, the entire herd from which these two animals were selected was examined for streptococcic mastitis by the blood-plate method at monthly intervals. From the very beginning of these tests, both animals were found to harbor *Str. agalactiae* in all quarters. When the treatment was started, one of the cows was at the beginning of the drying off period, while the second cow had calved one day prior to treatment. In these preliminary tests, only two of the infected quarters of each cow were treated, and small doses (20 cc. or approximately 7.5 Gm. of sulfanilamide) of the homogenized sulfanilamide-oil mixture were administered in order to determine the tolerance of the udder tissue for sulfanilamide, and also to ascertain whether injection of the drug into two of the infected quarters would diffuse in sufficient concentration to destroy the streptococci in the other two infected quarters. The drug was well tolerated by the gland and there was no udder reaction in either the dry or milking animals. The milk from the udders of both animals was normal and there was no noticeable decrease in the amount of milk flow. Even when the dose was increased to twice the amount originally injected, there was no indication of irritation. As is shown in table 2, the streptococci soon disappeared from the treated quarters. Although the milk from the untreated quarters contained sulfanilamide, indicating that the drug had diffused into the tissue of the nontreated quarters, the concentration in them was not sufficient to affect the streptococci present in any appreciable degree. The quarters which were left untreated at the beginning of the experiment were soon freed of the infection when treated with homogenized sulfanilamide. To date, nine months after the treatment, these two cows are still free from *Str. agalactiae* infection.

Following these preliminary experiments, the treatment with homogenized sulfanilamide-in-oil was given to all the streptococcic mastitis-infected animals in four large herds. Three of the herds were composed of Guernsey cattle and the fourth was a Holstein-Friesian herd. As a result of regular monthly tests for detecting cows infected with mastitis streptococci, the status of all animals in these herds was well established. *Str. agalactiae* infection was very prevalent in these herds.

Herd A was a purebred Guernsey herd consisting of 61 milking cows, of which 34 or 54 per cent had chronic streptococcic mastitis. Ten of the infected animals were eliminated from the herd because of brucellosis, thus leaving 23 cows to receive the intramammary oil-sulfanilamide treatment. Of the 23 cows treated, 22 were freed from *Str. agalactiae*, as shown in frequent tests of the milk by the blood-agar plate method. The animal classified as uncured in this group was removed from the herd because of brucellosis contracted before the mastitis treatment was complete. As shown in table 3, 13 cows were cured with one series of injections, one was cured with two series, and nine were listed as miscellaneous since some quarters required more treatments than others.

Herd B was a herd of 117 purebred Guernsey cows. At the time this treatment was instituted there were 37 animals infected with *Str. agalactiae*. As shown in table 3, 28 cows were cured with one series (four daily intramammary injections of 40 cc. each dose), three with two series of injections and six cows received more treatments in some quarters than in others. In the latter group two cows which were originally infected in all four quarters were cured in three quarters while the fourth quarter failed to respond in spite of four series of treatments. Although no definite explanation can be offered for failure of these quarters to respond, it is possible that the infection was either deep seated or that the strain of *Str. agalactiae* was "drug-fast."

Herd C consisted of 66 purebred Holstein-Friesian cows. When the treatment with oil-sulfanilamide mixture was started in this group, 34 animals had chronic streptococcic mastitis. Of these 33 were shedders of *Str. agalactiae* and one of *Str. uberis*. Since some

of these cows had large udders, it was necessary to increase the daily dose to as much as 80 cc. in some cases. In spite of the large doses given, there were no noticeable ill effects produced. Of the 34 animals treated, 31 were cured and 3 failed to respond in one quarter each, although the other treated quarters were cured.

Herds D and E were originally one herd of purebred Guernseys. A plan of segregation was put in operation and all the mastitis-positive cows were removed to a separate farm; this group became herd D, whereas the mastitis-negative group became herd E. For the past three years the animals in the latter herd have been tested for streptococcic mastitis at monthly intervals. With the exception of two cows from which *Str. uberis* has been isolated, all animals in this herd (about 70 cows) have remained free from *Str. agalactiae* during this entire period. Two cows infected with *Str. uberis* received four daily treatments which resulted in complete cure in both cases. Of the 11 infected cows that were originally removed to herd D, seven were available when this treatment was started. Although these cases

TABLE II—Effect of Sulfanilamide Homogenized in Liiquid Petrolatum in Treated and Untreated Quarters Infected with Streptococcus Agalactiae

Date	Milk Samples Examined for Streptococcus Agalactiae (Lancefield Group B)				Sm. Dosage—cc.			
	Right Rear	Right Fore	Left Rear	Left Fore	RR	RF	LR	LF
Cow F-29 Dry								
7/30/41	(x) α′-Str.	x) α′-Str.	(x) α′-Str.	(x) α′-Str.	20		20	
7/31/41	10 col. α′-Str	α′-Str.	14 col. α′-Str.	(x) α′-Str.	20		20	
8/ 1/41	Normal	(x) α′-Str.	Normal	(x) α′-Str.	20		20	
8/ 2/41	Normal	(x) α′-Str.	Normal	(x) α′-Str.	40		40	
8/ 4/41	Normal	(x) α′-Str.	Normal	(x) α′-Str.				
8/ 5/41	Normal	(x) α′-Str.	Normal	(x) α′-Str.				
8/ 6/41	Normal	(x) α′-Str.	Normal	(x) α′-Str.		20		20
8/ 7/41	Normal	5 col. α′-Str.	Normal	13 col. α′-Str.		20		20
8/ 8/41	Normal	2 col. α′-Str.	Normal	Normal		20		20
8/ 9/41	Normal	Normal	Normal	Normal		20		20
8/11/41	Normal	2 col. α′-Str.	Normal	Normal				
8/12/41	Normal	(x) α′-Str.	Normal	Normal		40		40
8/13/41	Normal	Normal	Normal	Normal		40		40
8/14/41	Normal	Normal	Normal	Normal				
8/15/41	Normal	Normal	Normal	Normal				
9/ 8/41	Normal	Normal	Normal	Normal				
9/12/41	Normal	Normal	Normal	Normal				
10/17/41	Normal	Normal	Normal	Normal				
1/15/42	Normal	Normal	Normal	Normal				
2/10/42	Normal	Normal	Normal	Normal				
3/13/42	Normal	Normal	Normal	Normal				
Cow F-83 Lactating								
7/28/41	(x) α′-Str.	(x) α′-Str.	(x) α′-Str.	(x) α′-Str.	20		20	
7/30/41	50 col. α′-Str.	(x) α′-Str.	Normal	(x) α′-Str.	20		20	
7/31/41	8 col. α′-Str.	(x) α′-Str.	Normal	(x) α′-Str.	20		20	
8/ 1/41	2 col. α′-Str.	50 col. α′-Str.	Normal	(x) α′-Str.	40			
8/ 2/41	Normal	(x) α′-Str.	Normal	(x) α′-Str.				
8/ 4/41	Normal	(x) α′-Str.	Normal	(x) α′-Str.		40		40
8/ 5/41	Normal	2 col. α′-Str.	Normal	15 col. α′-Str.		40		40
8/ 6/41	Normal	Normal	Normal	Normal		40		40
8/11/41	Normal	Normal	Normal	Normal				
8/15/41	Normal	Normal	Normal	Normal				
9/ 8/41	Normal	Normal	Normal	Normal				
9/12/41	Normal	Normal	Normal	Normal				
10/17/41	Normal	Normal	Normal	Normal				
1/15/42	Normal	Normal	Normal	Normal				
2/10/42	Normal	Normal	Normal	Normal				
3/13/42	Normal	Normal	Normal	Normal				

(x) = Innumerable colonies developed on blood-agar plate streaked with a 4 mm. loop of milk.
Sm. Dosage = Sulfanilamide homogenized in liquid petrolatum.

had previously been treated with colloidal silver oxide, they continued to harbor *Str. agalactiae*. Sixteen quarters were treated in this group with the oil-sulfanilamide method, and there were eight cured and eight uncured quarters. However, when the results are interpreted on the basis of the number of cows cured, there were only two. It may be mentioned in connection with this group that the amount of sulfanilamide given was less than that which is now recommended as standard treatment.

A summary of the results on 103 cases is presented in table 3. The data in this table show the number of cows and quarters treated and the number of series of treatments given. Thus, in herd A, there were 23 cows with 68 infected quarters treated. With the exception of one incomplete case, all were cured. To date, this entire herd is still negative for streptococcic mastitis. In herd B, in which 37 cows with 85 infected quarters were treated, there were 35 cows and 83 quarters cured. Of 34 cows with 92 infected quarters treated in herd C, 31 cows and 89 quarters responded to the treatment. In herd D, two out of seven cows and eight out of 16 quarters were cured; and in herd E, both animals which were infected with *Str. uberis* were cured.

It will be noted that when all animals on the four farms are grouped together, 89.3 per cent were cured and 10.7 per cent were listed as not cured. Furthermore, it will be seen that in the 103 cows, the number of infected quarters treated was 265, of which 251 (94.7%) were cured and 14 (5.3%) not cured. In considering these statistics it should be noted that some of the cases reported in this study were animals treated early in the investigation when the dose of the homogenized sulfanilamide-in-oil mixture employed was considerably less than that later regarded as the adequate dose.

EFFECT ON THE MAMMARY GLAND AND MILK PRODUCTION

The effect of homogenized sulfanilamide upon the secretory tissue of the gland and on milk production is in marked contrast to that produced by other antiseptics. Mammary tissue is sensitive. Chemicals ordi-

(*Continued on page 282*)

TABLE III—Detailed Analysis of 103 Cows Treated for Streptococcic Bovine Mastitis with Sulfanilamide Homogenized in Liquid Petrolatum

Herd		Courses of Treatments Received					Total No. Treated		Cured				Not Cured			
									Cows		Quarters		Cows		Quarters	
		1	2	3	4	Misc.	Cows	Qu.	No.	%	No.	%	No.	%	No.	%
A	Cows	13	1			9	23		22	95.6			1*	4.4		
	Qu.	44	16	7	1			68			67	98.5			1*	1.5
B	Cows	28	3			6	37		35	94.6			2	5.4		
	Qu.	66	13	4	2			85			83	97.6			2	2.6
C	Cows	19**		1		14	34		31	91.1			3	8.9		
	Qu.	58	19	9	6			92			89	96.7			3	3.3
D	Cows	2	5				7		2	28.5			5	71.5		
	Qu.	4	12					16			8	50.0			8	50.0
E	Cows	2***					2		2	100			0	0		
	Qu.	4***						4			4	100.0			0	0
Total							103	265	92	89.3	251	94.7	11	10.7	14	5.3

* = This animal was slaughtered because of Brucella infection before mastitis treatment was completed.
** = One animal in this herd was infected with *Str. uberis*. This cow is among those cured.
*** = Both animals in this herd were infected with *Str. uberis*.
Of 103 cows treated, 100 were infected with *Str. agalactiae* and three were infected with *Str. uberis*.

TABLE IV—Protocols of Typical Mastitis Cases Treat with Sulfanilamide Homogenized in Liquid Petrolatum

DATE	MILK SAMPLES EXAMINED FOR STR. AGALACTIAE (LANCEFIELD GROUP B)				SM. DOSAGE**—CC.			
	RIGHT REAR	RIGHT FORE	LEFT REAR	LEFT FORE	RR	RF	LR	LF
			Cow B-7					
10/10/41	(x) α′-Str.	(x) α′-Str.	(x) α′-Str.	(x) α′-Str.				
10/13/41					40	40	40	40
10/14/41	Normal	Normal	Normal	Normal	40	40	40	40
10/15/41	Normal	Normal	Normal	Normal	40	40	40	40
10/16/41	Normal	Normal	Normal	Normal	40	40	40	40
10/17/41	Normal	Normal	Normal	Normal				
10/20/41	Normal	Normal	Normal	Normal				
12/ 1/41	Normal	Normal	Normal	Normal				
			Cow B-14					
10/17/41	Normal	Normal	(x) α′-Str.	Normal				
10/20/41							40	
10/21/41	Normal	Normal	10 col. α′-Str.	Normal			40	
10/22/41	Normal	Normal	4 col. α′-Str.	Normal			40	
10/23/41	Normal	Normal	3 col. α′-Str.	Normal			40	
10/28/41	Normal	Normal	50 col. α′-Str.	Normal				
10/29/41							40	
10/30/41	Normal	Normal	3 col. α′-Str.	Normal			40	
10/31/41	Normal	Normal	Normal	Normal			40	
11/ 4/41	Normal	Normal	Normal	Normal				
11/25/41	Normal	Normal	Normal	Normal				
12/16/41	Normal	Normal	Normal	Normal				
2/ 2/42	Normal	Normal	Normal	Normal				
3/ 2/42	Normal	Normal	Normal	Normal				
			Cow F-78					
9/15/41	(x) α′-Str.	(x) α′-Str.	(x) α′-Str.	(x) α′-Str.				
9/18/41					40	40	40	40
9/19/41	50 col. α′-Str.	18 col. α′-Str.	30 col. α′-Str.	20 col. α′-Str.	40	40	40	40
9/20/41	10 col. α′-Str.	2 col. α′-Str.	5 col. α′-Str.	8 col. α′-Str.	40	40	40	40
9/22/41	7 col. α′-Str.	Normal	2 col. α′-Str.	Normal				
9/29/41	(x) α′-Str.	8 col. α′-Str.	(x) α′-Str.	(x) α′-Str.				
10/ 1/41					40	40	40	40
10/ 2/41	Normal	Normal	Normal	3 col. α′-Str.	40	40	40	40
10/ 3/41	Normal	Normal	Normal	Normal	40	40	40	40
10/ 4/41	Normal	Normal	Normal	Normal	40	40	40	40
10/11/41	Normal	Normal	Normal	Normal				
11/ 3/41	Normal	Normal	Normal	Normal				
12/ 2/41	9 col. α′-Str.	Normal	Normal	Normal	40			
12/ 3/41	Normal	Normal	Normal	Normal	40			
12/ 4/41	Normal	Normal	Normal	Normal	40			
12/ 5/41	Normal	Normal	Normal	Normal	40			
12/11/41	Normal	Normal	Normal	Normal				
1/16/42	Normal	Normal	Normal	Normal				
4/20/42	Normal	Normal	Normal	Normal				

*(x) = Innumerable colonies developed on blood-agar plate streaked with a 4 mm. loop of milk.
Sm. Dosage = Sulfanilamide homogenized in liquid petrolatum.

In table IV, protocols are presented on three cases in the several groups with reference to the number of treatments required to bring about a complete cure. Cow B-7 was cured by one course of treatment; in fact, this animal was cured by the first injection. Cow B-14 required two courses of treatment; and cow F-78 three courses before she was cured in one infected quarter. The other infected quarters required fewer treatments.

narily considered nonirritating may produce a marked reaction when introduced into the udder. Little, Dubos and Hotchkiss[19] report that Ringer's solution proved even more irritating than distilled water as a vehicle for gramicidin. These workers conclude that ". . . too extensive treatment with gramicidin in any form or possibly with other bactericidal agents may result in damage to the secretory tissue." Weirether *et al.*[20] report that intramammary injections of colloidal silver oxide produced marked inflammation of the udder accompanied by abnormal milk secretion. Intramammary injection of homogenized sulfanilamide-in-oil, used in more than 100 udders, was found to be nonirritating. Reactions occurred in only two animals. These reactions were mild and occurred following the first injection. They occurred in but one quarter in each case receiving the homogenized sulfanilamide in all quarters. The reaction consisted of a slight swelling of the quarter which was soft and not hot or painful. These swellings were observed 24 hours after the initial injection and were not considered serious enough to interfere with the second injection. Both animals were, therefore, reinjected with the usual dose in the slightly swollen quarters. When next examined 24 hours later, the swelling had entirely subsided.

When large doses of sulfanilamide are administered orally a decrease in milk production has been observed. This has also been reported by other workers, notably Gildow, Fourt and Shaw,[4] Hoge *et al.*,[5] and Stevenson.[8] In the cases described herein, the homogenized sulfanilamide treatment had no effect on the milk production during or after treatment. The treatment was given to some cows which at the time were on test without any decrease in the amount of milk flow. In cases where the milk was granular or flaky previous to the treatment, the milk became normal in appearance and usually returned to normal flow soon after three or four injections had been administered. This treatment has been employed in acute, chronic and subclinical mastitis with satisfactory results. It may be used in dry or lactating animals. In subclinical cases giving apparently normal milk the homogenized sulfanilamide-in-oil produces no changes in the physical appearance or quantity of milk secreted.

HOMOGENIZED SULFATHIAZOLE AND SULFANILAMIDE IN STAPHYLOCOCCIC MASTITIS

Staphylococcic mastitis ranks next in importance to streptococcic mastitis. Herds have been observed in which staphylococcic mastitis was more serious than streptococcic mastitis. The clinical similarity of the two types has been reported by several workers. Minett[21] reported that ". . . cases of mastitis, acute and chronic, caused by pathogenic staphylococci (*S. aureus*) have been found to be of such a nature as to be confused with mastitis caused by streptococci . . ." It has been observed by this group that, clinically, mastitis caused by *Staphylococcus aureus* is indistinguishable from that caused by streptococci, except possibly those very acute cases accompanied by extreme toxemia or udder necrosis. This organism may cause a chronic mastitis very much like that produced by *Str. agalactiae* or it may cause a mild or acute mastitis. It has been observed that in herds where acute staphylococcic mastitis occurs quite frequently at calving time, the cows which develop the disease usually were carrying pathogenic strains of *S. aureus* in their udders prior to calving.

Pathogenic strains of *S. aureus* differ from the harmless nonpathogenic strains, which are present in variable numbers in the udders of practically all cows, in that they produce powerful toxins. In sufficient amounts these toxins are lethal for the animal and are capable of causing necrosis of the udder. The reason that such cases are more prevalent at calving time may be due to the increased amount of carbon dioxide in the tissues at that time. It is well established that an increase in carbon dioxide is necessary for the production of these toxins and so conditions are ideal for the organisms to produce them.

From experience in treating these cases, it is believed that it is better to try to detect them before the cows freshen and treat

them immediately rather than wait until the cows have freshened and symptoms of mastitis have appeared. By treating them at this time it may be possible to prevent serious mastitis at calving time. Furthermore, such organisms, if permitted to remain in the udder, are not only potential sources of acute mastitis throughout the lactation period, but also will set up chronic inflammatory changes comparable to those produced by streptococci. Such organisms in the milk constitute a public health menace.

The method of diagnosis employed in this study was similar to that for streptococci. *S. aureus* grows readily on the tryptose blood-agar plates. In differentiating the pathogenic and nonpathogenic strains, pigment production is of little or no significance. Pathogenic strains are usually hemolytic but not always. It has been shown that pathogenic strains produce coagulase which will coagulate citrated blood. Based upon studies on mastitis in heifers[22] and the work of Plastridge *et al.*,[23] the coagulase test, when made on rabbit plasma, is considered the most valuable in separating pathogenic from nonpathogenic staphylococci.

Natural recovery may occur in staphylococcic mastitis, particularly in mature cows, as reported by this group[22] and by Jones,[23] but such animals appear to be the exception. Cows which are constant shedders of pathogenic strains of *S. aureus* or animals with clinical mastitis caused by this organism should receive treatment. This organism requires a larger dose of the homogenized sulfanilamide-in-oil mixture for its destruction than the mastitis streptococci. It was found that 80 cc. doses of the standard preparation destroyed staphylococci in all cases studied.

In early studies on staphylococcic mastitis in this laboratory, sulfathiazole in liquid petrolatum was employed as the therapeutic agent. This drug proved satisfactory for destroying the infection, and smaller doses are required than with sulfanilamide. However, it is objectionable for two reasons, namely: it is more expensive than sulfanilamide, and it causes the formation of hard curds of casein difficult to remove which temporarily plug the teat duct.

In the twelve cases treated with sulfanilamide-in-oil, excellent results have been obtained. In advanced cases, however, where toxemia and udder necrosis are well established, the treatment was of no value. Three cases showing marked toxemia without evidence of udder necrosis were treated successfully. These cases made rapid recoveries with the temperature and appetite returning to normal, and the milk becoming normal and free of infection within a few days. In chronic and subclinical cases of staphylococcic mastitis, satisfactory results were obtained with the procedure recommended for treating streptococcic infection, except that the standard dose of 40 cc. is increased to 80 cc. When used in this amount and repeated daily for four days, it is indicated that homogenized sulfanilamide-in-oil is as effective for staphylococcic mastitis as the smaller dose for streptococcic mastitis. The conclusion is based upon the study of but a small number of cases. This phase of the problem is now under investigation.

SUMMARY AND CONCLUSIONS

This report deals with the chemotherapeutic properties of sulfanilamide when administered directly into the bovine mammary gland for the treatment of streptococcic and staphylococcic mastitis. Light liquid petrolatum has been found to be a suitable vehicle for incorporating and administering the drug. A method for the preparation of the homogenized sulfanilamide and a procedure for intramammary injections are described.

In streptococcic mastitis the dosage recommended for an average size udder is 40 cc. of the homogenized product given daily for four days. If the infection persists, the treatment should be repeated and the daily dose may be increased to 80 cc. with safety. This applies both to lactating and nonlactating cows.

In staphylococcic mastitis the dose recommended is 80 cc. of the product given daily for four days and repeated if indicated.

The homogenized sulfanilamide as used in these experiments was well tolerated and readily absorbed by the mammary gland. It can be used in acute, subclinical and chronic forms of mastitis. No contraindications have been found. There was no decrease in milk production during or after treatment.

One hundred and three cows were treated for streptococcic mastitis by intramammary injections with homogenized sulfanilamide. One hundred of these were infected with *Streptococcus agalactiae* and three were infected with *Streptococcus uberis*. Based on bacteriological findings, cures were effected in 92 cows (89.3%), and partial or no improvement in 11 cows (10.7%). Breaking down the results into quarters treated, of the total 265 quarters, 251 (94.7%) were cured, and 14 (5.3%) were not cured.

In acute and chronic mastitis caused by pathogenic staphylococci, promising results were obtained in preliminary trials from intramammary use of homogenized sulfanilamide.

Sulfathiazole homogenized in liquid petrolatum was employed in several cases of acute and chronic staphylococcic mastitis. This drug, although capable of destroying the infection within the udder, is objectionable owing to the formation of plugs which temporarily occlude the teat duct.

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Significance of Inclusion Bodies of Distemper*

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THE PRESENCE OR absence of inclusion bodies in certain epithelial cells has recently become a means of diagnosing distemper. Identification of this disease by the evaluation of the history, clinical symptoms, autopsy findings and, in some cases, ferret-inoculation tests has come to be regarded as slow and uncertain. Growing reliance on the inclusion-body picture, both from sectioned and smear preparations, tends to depreciate the importance of the older diagnostic aids. It also presents possibilities for error in diagnosis through failure to differentiate inclusion-like bodies not representative of clinical distemper.

This discussion is confined to distemper inclusion bodies of foxes and minks. Both of these species are raised domestically in numbers large enough to constitute a valuable national economic entity. The usual management practices involve the concentration of large numbers of these animals in small, fenced-in, ranch areas with subdivisions for single animals, pairs, families, and variable sized groups. This provides an animal concentration favorable for the rapid spread of a contagion. The early and accurate diagnosis of a disease such as distemper becomes a problem of paramount importance. One or a few animals are willingly sacrificed for laboratory diagnosis to provide information that will help to prevent the spread of the disease and thus protect the mass population on the ranch. This is quite different from the case of the individually owned, family dog, in which diagnosis is incumbent during the clinical phases of the disease to provide opportunity for restoration to health. These circumstances point to the reason that distemper inclusion bodies are more studied in foxes and minks than in dogs and other susceptible species.

Distemper inclusion bodies are normally found in epithelial cells of animals sick in the advanced stages or dead from the disease. The further the disease has advanced, the more often inclusion bodies are apt to be present. They occur more frequently and more abundantly in the bronchial and bladder epithelium than in other epithelial tissues (fig. 1).

Distemper inclusion bodies usually are located in the cytoplasm, though occasionally they occur in the nucleus. Typical inclusions vary in size, shape, and number per cell; they have definite boundaries and may contain characteristic refractive granules or vacuoles. If numerous characteristic inclusion bodies are present in a preparation, a diagnosis can be made immediately on the basis of the observation if it is consistent with the available information on the case.

Preparations for the observation of inclusion bodies are obtained by two methods: (1) the smear method and (2) sectioning. Smears are prepared directly from the tissues at the time of autopsy by rolling a cotton swab over the epithelium of the desired tissue and then over a clean slide. The cotton swab is made by wrapping a piece of cotton over one end of a toothpick. After air drying for 10 to 15 minutes, the slides are ready to stain. This method does not injure the cells as much as when they are scraped off with a scalpel and smeared on a slide. In any event, some cells will be broken in a smear, but many will come through intact.

Material for sectioning also is collected at the time of autopsy. Small pieces of the tissues are placed in a fixative, then dehydrated, cleared and embedded in paraffin from which thin sections are cut and prepared for staining. This method takes much longer than the smear technique, but the cells are held in their original position. Often, this longer method aids in deciding

*From the Department of Veterinary Science, University of Wisconsin; presented before the Section on Research at the seventy-eighth annual meeting of the American Veterinary Medical Association, Indianapolis, Ind., August 11-15, 1941.
Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

a questionable diagnosis. Delafield's hematoxylin with an eosin counter stain has been used effectively in this work, although several other stains have been tried with varying degrees of success. The hematoxylin stains the cell walls, nuclei, and cytoplasm while the eosin is absorbed by the inclusion bodies. This combination gives a good contrasting stain. Red blood cells also take on the eosin stain, but their regular size and shape as well as their location make it easy to distinguish them from a typical inclusion body. They are not found in the cytoplasm

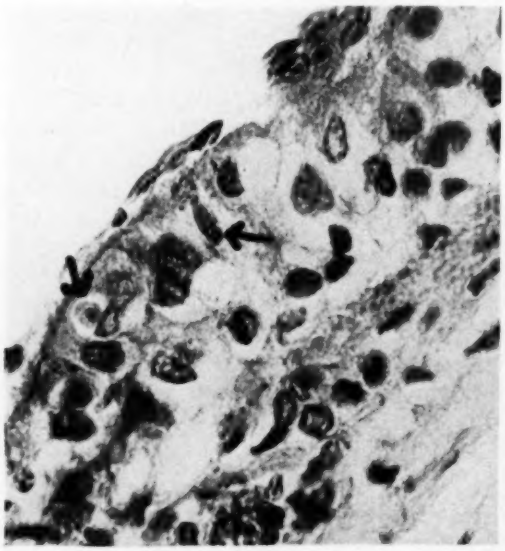

Figure 1—Normal inclusion bodies in bronchial epithelium of fox.

of epithelial cells and seldom occur just over or under this area.

From the brief review of this method, it may appear that the diagnosis of distemper by the use of inclusion bodies is a "cut and dried" procedure. However, confusing situations sometimes occur and it is these conditions which are to be emphasized for the consideration of veterinarians and technicians who are called upon to make a diagnosis for or against distemper.

Approximately 646 fox and mink specimens have been examined at the University of Wisconsin laboratories for distemper inclusion bodies. Eighty-six were exposed to the distemper virus experimentally; the other 560 were sent or brought in by fur farmers in Wisconsin and other fur-raising states. Of these 560 animals, 15 foxes and 41 minks were definitely diagnosed as having distemper by means of the inclusion body picture together with the history, clinical symptoms, and postmortem findings.

In addition, from these 560 animals, 31 specimens showed "suspicious" bodies on either the smears or sectioned material. These bodies might be mistaken for distemper inclusions by an observer if the diagnosis were based on the presence of inclusion bodies alone. Twenty-two foxes and nine minks were included in the group. Both smears and sectioned material were examined from confusing cases as well as from many other specimens.

Two kinds of distemper inclusion-like bodies were found in epithelial tissues, where distemper inclusions most frequently occur. Such bodies, however, did

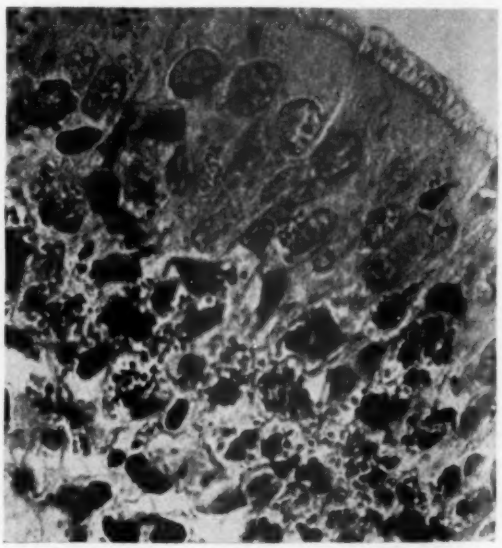

Figure 2—Inclusion-like bodies in epithelium and submucosa of bronchus of healthy fox.

not represent distemper in its clinical form.

One type encountered, produced small groups or clusters of eosinophilic bodies in the cytoplasm of epithelial cells from animals with negative histories, clinical symptoms, and autopsy findings for distemper. Animals from one particular ranch where inclusion-like bodies occur have been ob-

served for two years. The observations were made on foxes killed at pelting time. These animals had no history or outward symptoms of distemper. They were in a fat condition and appeared to be in good health. However, most of them did have a history of bladder trouble. The smears showed numerous, scattered, small, eosinophilic bodies, most of them regular but varying

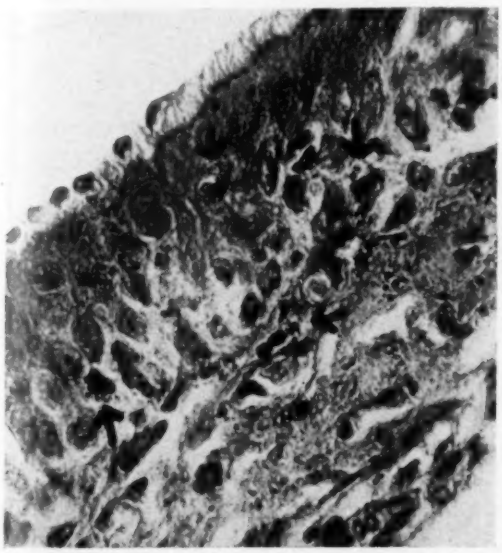

Figure 3—Inclusion bodies in bronchial epithelium from fox exposed to distemper virus.

in size. Sectioned material revealed these bodies to be in groups in the cytoplasm of epithelial cells and also cells of the submucosa. Some of these inclusion-like bodies appeared to have refractive granules within them (fig. 2).

The typical inclusion bodies mentioned earlier in this paper were those found in animals dead from distemper. They were comparatively large and occurred singly in different cells, or one or more were located in different parts of the protoplasm of the same cell. In other words, they did not occur in clusters or small groups. The experimental foxes which were exposed to the distemper virus and killed at different stages of the disease showed small eosinophilic bodies either in isolated groups or together with characteristic inclusion bodies. The case which has no history of distemper, but in which inclusion-like bodies are found, presents an important problem in diagnosis. The occurrence of groups of small eosinophilic staining bodies has not been confined to one ranch, but has appeared in animals from several other ranches which had no history of distemper (fig. 3).

The other type of inclusion-like body appeared more often in smears than in sectioned material and was found on slides from both foxes and minks. These bodies were frequently dissociated from the cell cytoplasm, although their appearance was similar to distemper inclusions. They varied in size and often possessed refractive granules. They took on the definite contrasting eosin stain in every case. When tissues from these animals were sectioned, inclusion-like bodies were rarely found. When characteristic distemper inclusion bodies were found either in smears or sec-

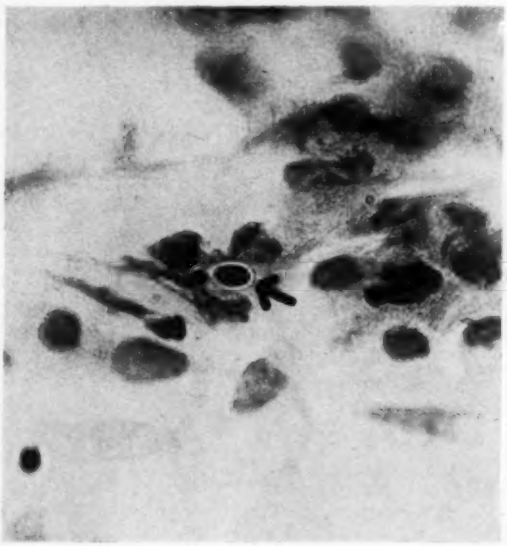

Figure 4—Inclusion-like body from tracheal smear of red fox.

tioned material, they usually occurred in greater numbers than in the above-described conditions (fig. 4).

Still another condition may arise which might be confusing in making a diagnosis from smears alone. Sometimes, the inclusion bodies in a particular animal may be very large and granular. When cells containing such inclusions are smeared on a slide, the cytoplasm of these cells and their

inclusions may be broken so that the inclusions lose their identity and the material on the slide appears granular. This condition is more apt to happen when the cells are scraped from the tissues with a scalpel and smeared on a slide than when a cotton swab is used. Large inclusion bodies that have been broken and mixed with other material do not take on the contrasting stain in the characteristic way, thus making it possible to confuse the granules with some outside factor. Failure to identify the inclusion bodies in smears under these conditions can be rectified by checking the sectioned material (fig. 5).

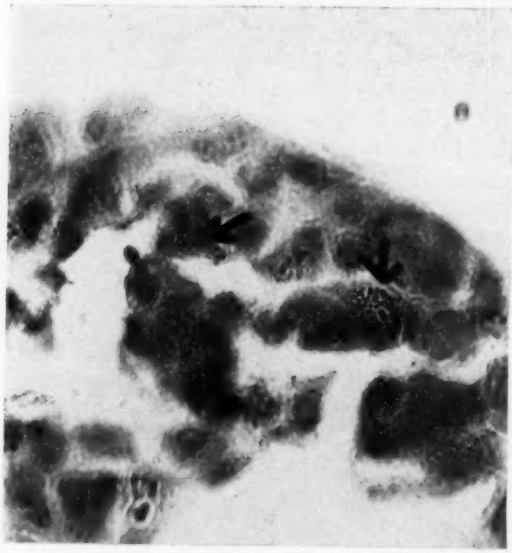

Figure 5—Large broken down inclusion bodies in bladder epithelium smear of mink.

SUMMARY

Early and accurate diagnosis of distemper is essential on fox and mink ranches. Distemper inclusion bodies provide an invaluable aid in diagnosing distemper, yet there are conditions when the presence of several kinds of inclusion-like bodies confuses the diagnosis. It is essential, therefore, that the diagnosis be based on history, clinical picture, postmortem gross appearance, and presence of characteristic inclusion bodies.

Serial Passage of Lymphomatosis-Osteopetrosis in Chickens*

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STUDY OF THE avian leucosis complex as outlined in the program of the U. S. Regional Poultry Research Laboratory embraces attempts to propagate in chickens several different strains of the agent or agents of the complex. Four of six inoculums obtained at the beginning of the experimental work in 1939 induced characteristic manifestations of the avian leucosis complex when injected into young White Leghorn chickens.

The results obtained with the agent designated strain 3, lymphomatosis-osteopetrosis, form the basis of this report. This inoculum originated from three cases of lymphomatosis in White Leghorn hens which were supplied by Dr. A. J. Durant of the University of Missouri.

Strain 3 differs from two other strains of lymphomatosis under study. In addition to the apparently typical visceral, neural, and ocular forms of lymphomatosis manifested by all of these strains, there has occurred in birds inoculated with strain 3 the hypertrophic osteopathy described and termed osteopetrosis gallinarum by Jungherr and Landauer.[1] These authors made a comprehensive review of the literature dealing with somewhat similar pathologic bone conditions in various species and a complete description and discussion of osteopetrosis gallinarum.

The tentative classification of the avian leucosis complex[2] includes osteopetrotic manifestations as an expression of lymphomatosis. For the purpose of comparison in this report, and since its possible indentity as a manifestation of lymphomatosis will require further confirmation, osteopetrosis is considered separately from lymphomatosis.

SOURCE AND PREPARATION OF INOCULUM

The three hens (879, 880, and 881) which provided the original strain 3 inoculum were obtained by Dr. Durant from a farm flock in Missouri in which there had been a high incidence of lymphomatosis, the ocular type predominating. All three specimens showed varying degrees of bilateral lymphomatotic infiltration of the irises. In addition to eye lesions, hen 881 showed spastic flexor paralysis of the left leg. Necropsy examination revealed a large, circumscribed, irregular, cylindrical mass of tissue, approximately 15 x 35 mm. in diameter, surrounding and involving the midfemoral portion of the left sciatic nerve. The mass, yellowish to pinkish white, was relatively dense and somewhat more firm than the usual diffuse lymphomatotic involvement of the sciatic nerves. There was no gross evidence of osteopetrotic involvement of the bones in these specimens, although typical osteopetrosis occurred in birds of the first and subsequent passages. The findings on these individuals and on donors of inoculums for subsequent passages are summarized in table I.

Whole blood was used almost exclusively for inoculating the various series of birds. It was collected aseptically from selected typical specimens by cardiac puncture. Heparin in Locke's solution was employed as an anticoagulant. As a rule, inoculations were made on the same day, and in no case later than 72 hours after the blood was drawn. Blood held over from one day to the next was stored at 4 to 8 C.

SOURCE AND INOCULATION OF CHICKS

The 1939, or first passage, chickens represented approximately equal numbers of individuals from nine different strains or flocks of White Leghorns. Pedigreed hatching eggs were set at weekly intervals over a period of four weeks during April and May 1939. Each hatch was divided on the basis of strain and family into three series or groups: one for inoculation, an uninoculated group to serve as pen-contact controls to the inoculated lot, and a third non-

*From the East Lansing Regional Poultry Research Laboratory, Bureau of Animal Industry, U. S. Department of Agriculture; presented before the Section on Poultry at the seventy-eight annual meeting of the American Veterinary Medical Association, Indianapolis, Ind., August 11-15, 1941.

[1] Jungherr, E., and Landauer, W.: Studies on fowl paralysis. A condition resembling osteopetrosis (marble bone) in the common fowl. Connecticut State Coll. Bul. 222 (1938), pp. 1-34.

[2] Tentative pathologic nomenclature. Am. J. Vet. Res., ii (1941), p. 116.

contact control group. The first two series were started and reared in the inoculation or infection unit of the plant while the last-mentioned group was brooded and reared in isolation in the control part or unit of the laboratory plant. All of the stock was maintained in complete confinement.

The chicks used in 1940 were the progeny of 1939 stock matings from the control-unit breeding pens. The offspring of 1940, as well as of 1939, control unit stock constituted the population for the early 1941 inoculations.

All inoculated chicks of the 1939, or first passage, series received the composite blood of hens 879, 880, and 881. At the time of inoculation, these chicks represented ages of 1, 8, 15, and 22 days. All chicks for subsequent passages were 3 weeks or less of age and the majority less than 1 week.

Fig. 1—Advanced clinical osteopetrosis bird B509A, 4th passage—donor for 5th passage. 92 days of age.

The usual route of injection was the brachial vein of the wing. A small percentage of young chicks, in which attempted intravenous inoculation was unsuccessful, were injected intracardiacally. Other routes of introducing inoculum were utilized in several small groups of chicks. The quantity of inoculum for each chick was seldom greater than 0.5 cc., all of which was given in one injection.

OBSERVATIONS AND EXAMINATIONS

The experimental birds were observed frequently and were kept under conditions of good sanitation and management in complete confinement. Individuals in the advanced or moribund stage of disease were removed for clinical and necropsy examinations. Frequent attention by the caretaker served to reduce the number of cannibalized and decomposed specimens.

Tissues were collected routinely at necropsy for histologic processing and study unless the carcass was badly decomposed or parts destroyed through cannibalism or other cause. However, bone specimens were not processed routinely. Total and differential blood counts, and in some cases hemoglobin determinations, were made on live birds. Differentiation into lymphomatosis and osteopetrosis positive groups was made on the basis of gross and microscopic interpretations. However, histologic examinations of a considerable number of specimens, including passage 1, have not been completed. Some birds of the first population are still living, as are also quite a large proportion in the fifth and sixth passage groups inoculated after January 1, 1941. The minimum period of observation, unless death intervened, was approximately six months. Elimination of some of the stock at the end of the six-month period following inoculation was necessary in several instances because of inadequate pen space.

RESULTS

The results of serial passage of strain 3 are recorded in table II. While the incidence of lymphomatosis in the control birds, both in the contact and non-contact groups, was over 20 per cent, it was twice as great in the inoculated stock. Evidence of inoculation transmission may be adduced from the significant increase in lymphomatosis and the occurrence of osteopetrosis only in the inoculated and not in the control stock. The possibility that some early, mild cases of osteopetrosis were not detected in the 1939 population is recognized. Our later observations emphasized that periodic examination of live

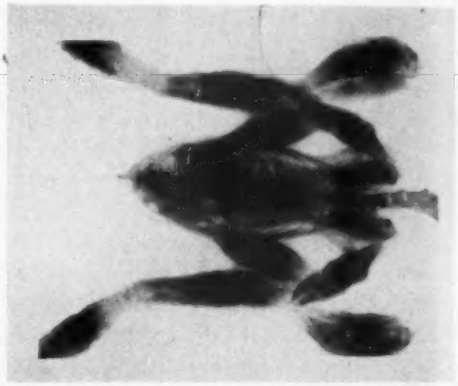

Fig. 2—Gross skeletal changes of osteopetrosis. (X-ray taken of bird 509A at 138 days of age.)

TABLE I—Data on Donors for Serial Passage of Strain 3, Lymphomatosis—Osteopetrosis

Passage No.	Donor No.	Donor's Age in Days	Diagnosis
1	879	Mature	Lymphomatosis—ocular
1	880	Mature	Lymphomatosis—ocular
1	881	Mature	Lymphomatosis—ocular and neural
2A	8A 751 R	228	Lymphomatosis—neural
2B	2A 122 P	228	Lymphomatosis—visceral
3A	B 11 A	73	Lymphomatosis—ocular
3B	B 5 C	133	Lymphomatosis—visceral, and osteopetrosis
3C	B 47 A	168	Lymphomatosis—neural
3D	B 55 A	209	Lymphomatosis—ocular
4A	B 5 E_4	155	Lymphomatosis—osteopetrosis
4A	B 36 G_3	93	Lymphomatosis—visceral, and osteopetrosis
5	B 8 A_5	83	Lymphomatosis—visceral, neural
5	B 8 B_5	146	Lymphomatosis—visceral, and osteopetrosis
5	B 8 D_5	190	Lymphomatosis—visceral, and osteopetrosis
5	B 8 F_5	155-174*	Lymphomatosis—visceral, and osteopetrosis
5	B 8 G_5	97-174*	Lymphomatosis—visceral, ocular, and osteopetrosis
5	B 309 A	61-100*	Lymphomatosis—visceral, and osteopetrosis
6	C 723 F_2	47	Lymphomatosis—neural
6	C 123 O	30-57*	Trauma and cannibalism
6	C 162 N	30-57*	Living (clinical osteopetrosis)
6	C 92 N	30-57*	Living
6	B 484 I	11	No visible lesions
6	B 517 A	11	No visible lesions

*Served as donors during the age period indicated.

birds and complete skeletal examination at autopsy are necessary to avoid overlooking mild or obscure cases. After osteopetrosis was first detected in serial passage of the strain 3 agent, Dr. Durant reported the condition in stock which he had inoculated with material originating from the same farm flock which provided the inoculum for initiating this passage.

With the use of chickens 1 to 22 days of age at inoculation, there appeared to be no significant differences associated with age at inoculation and the time elapsing before lymphomatosis and osteopetrosis appeared. One chick of the fifth serial passage inoculated at 21 days of age was observed to show definite clinical osteopetrosis of the shanks 57 days later. At the other extreme, one male of the second passage showed slight osteopetrosis 300 days after inoculation and mild to moderate lesions at death on the 371st day. The earliest age at which lymphomatosis in any form was detected in these passages was 35 days. One chick of the second passage, inoculated at 3 days of age, died of neural lymphomatosis 35 days later.

Marked variations were observed among osteopetrotic cases in the time of development and the extent of the osteopetrotic lesions. In some individuals the osteopathy was detected only on autopsy following death due to some form of lymphomatosis or to miscellaneous causes. In figure 1 is

TABLE II—Results of Serial Passage of Strain 3, Lymphomatosis—Osteopetrosis

Date of Hatch	Passage No.	No. Birds	No. Neg.	No. Pos.	No. Pos. Visceral*	No. Pos. Neural*	No. Pos. Ocular*	No. Pos. Osteopet.*
5/15-6/5/39	1	550	306	244	140	91	32	25
5/15-6/5/39	Controls—contact	340	266	74	55	20	7	0
5/15-6/5/39	Controls—non-contact	382	296	86	53	23	16	0
1/30/40	2A	31	25	6	1	5	0	0
1/22/40	2B	80	45	35	22	9	3	6
3/18/40	3A	13	9	4	2	2	0	0
6/4/40	3B	13	5	8	8	0	0	0
6/18/40	3C	4+	0	4	0	1	0	3
8/12/40	3D	21	3	18	17	0	0	8
10/28/40	4A	10	1	9	7	2	0	5
1/23/41	4B	3+	0	3	2	0	0	1
10/28/40-1/13/41	Controls 2-4P	14	11	3	1	2	0	0
1/30-4/28/41	5A-S	110	86	24	2	0	0	24
3/6-4/11/41	6A-G	49	43	6	0	1	0	8
1/30-4/28/41	Controls 5-6P	39	38	1	0	0	0	1
Total Inoc.		884	523	361 (40.8)	201 (22.8)	111 (12.6)	35 (4.0)	80 (9.0)
Total Control		775	611	164 (21.2)	109 (14.1)	45 (5.8)	23 (3.0)	1 (0.13)

*Totals include combinations of several forms of lymphomatosis and osteopetrosis.
†Inoculated as embryos.

shown a bird with enormous and extensive osteopetrosis at 92 days of age. The gait was markedly stilted and there was little inclination to move. The bird appeared definitely anemic. This specimen revealed metatarsal hyperpyresis and enlargement, apparently typical of clinical osteopetrosis at about a month of age. On palpation, marked enlargement of the shafts of other long bones could be detected.

Data on the occurrence of lymphomatosis and osteopetrosis in the several strains of birds comprising the first passage stock suggest that greater differences in susceptibility to osteopetrosis than to lymphomatosis existed among the strains of chickens used (table III). The incidence of osteopetrosis ranged from 0 to 12.7 per cent, that of lymphomatosis from 32.7 to 54.5 per cent. Data from special passages suggest the existence of family differences which would account in part for apparent

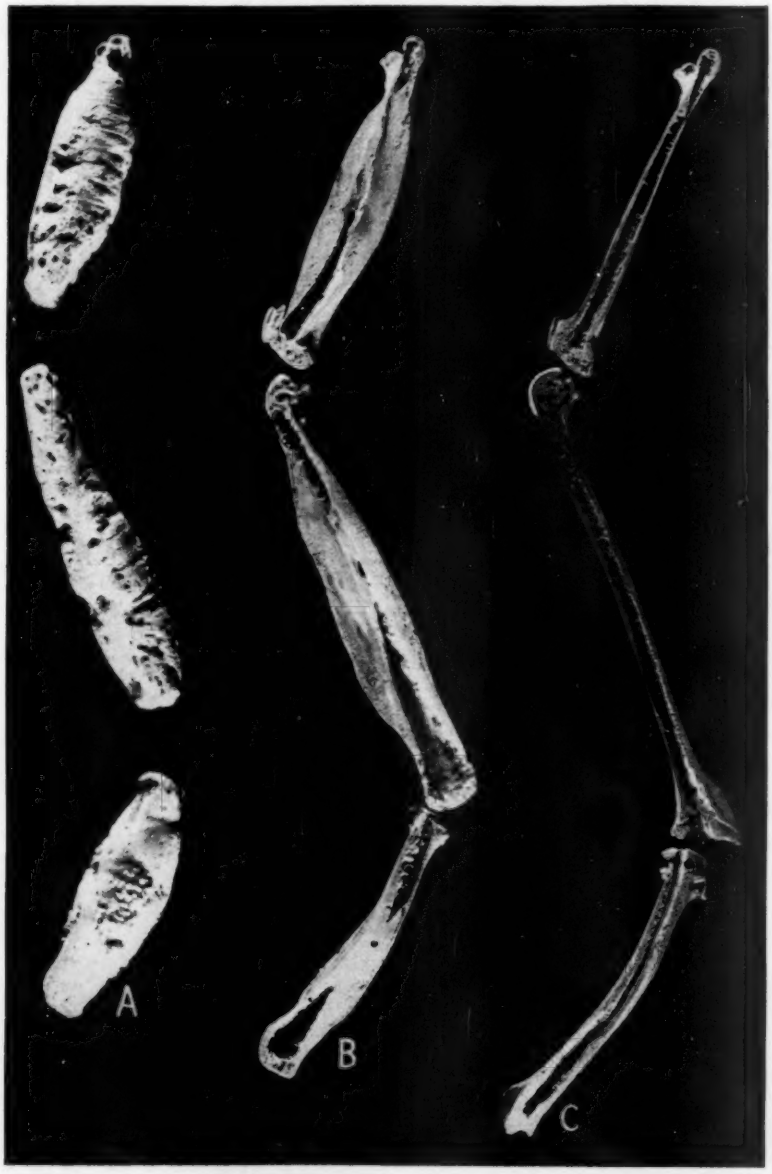

Fig. 3—Leg bones of: (a) B509A sacrificed in moribund state at 138 days of age. (b) A less marked case of osteopetrosis, B5C (137 days) sectioned longitudinally to show impingement and occlusion of marrow cavity. (c) A normal bird, 6A471A (422 days) sectioned longitudinally.

strain differences in osteopetrotic diathesis.

The occurrence of osteopetrosis, according to sexes, in the 1939 population is of interest in view of the fact that Pugh[3] observed an apparently similar condition only in males, and Jungherr[4] stated that the disease "affects various breeds and both sexes but especially males of the Frizzle variety." However, Jungherr and Landauer[1] reported an approximately equal sex distribution.

In the several serial passages of strain 3 inoculum, the male/female ratio of osteopetrosis for the 25 affected birds in the first passage was 17 to 8. This represented an incidence of 9.6 per cent of the 176 males and 3.0 per cent of the 267 females of the population. This included chicks which lived to at least 30 days of age and thereafter were held for the minimum 6-month observation period unless death intervened. In contrast, the incidence of lymphomatosis in this population was 46.8 per cent of the males and 51.7 per cent of the females.

In the third passage birds, it will be noted that osteopetrosis was not detected in series 2A, 3A, and 3B (table II). The etiologic agent or propensity for production of osteopathy may have been latent or overt in these passages, as well as in the original donors, only to remanifest itself in subsequent passages. This possibility was suggested by Jungherr and Landauer.[1]

The results of the fifth and sixth passages are not complete inasmuch as the inoculations were carried out during 1941 and in some series the observation periods do not exceed 60 days.

PATHOLOGY*

The gross and microscopic skeletal lesions observed in birds inoculated with strain 3 resembled essentially the lesions described by Jungherr and Landauer.[1] In some cases, there was an enormous enlargement in the diameter of the long bones of the extremities. Occasionally, in the severely involved cases, the bones of the pelvis, sternum, clavicle, coracoid, and scapula also were considerably enlarged (figs. 1 and 2). Typical involvement of the tarso-metatarsus (fig. 4) was characterized by augmented cellular activity of the periosteal region together with the presence of irregular, large blood spaces. Jungherr and Landauer[1] observed only relatively mild osseous enlargements in their inoculated birds, while the severe or massive involvements occurred only in the spontaneous cases.

TABLE III—Lymphomatosis—Osteopetrosis in Passage I Stock

Strain	No. Inoc.	Cases Showing Lymphomatosis		Cases Showing Osteopetrosis	
		No.	%	No.	%
1	58	24	41.4	0	0
2	52	17	32.7	0	0
3	66	36	54.5	4	6.1
4	59	22	37.3	3	5.1
5	63	29	46.0	8	12.7
6	69	23	33.3	3	4.3
7	59	26	44.1	2	3.4
8	61	24	39.3	2	3.3
10	63	32	50.8	3	4.8

Leucemic blood changes were not found in the limited number of cases of osteopetrosis studied. Anemia, secondary to the osteopetrotic involvement, was frequently observed. The progressively obliterative encroachment upon the marrow cavity probably may tend to impair hematopoiesis in severe cases of osteopetrosis. With few exceptions, the cases of osteopetrosis subjected to complete microscopic examination showed lesions of visceral and/or neural lymphomatosis. The lesions in the liver varied from extensive lymphomatotic involvement, apparent grossly, to relatively small focal aggregations of pathologic cells visible only microscopically.

Lymphomatotic lesions of the spleen, heart, kidney, adrenals, pancreas and gonads, while not as common as the liver involvement, varied to the same extent. The parathyroids were not observed to be

[3]Pugh, L. P.: Sporadic diffuse osteopetrosis of fowls. Vet. Rec., vii (1927), pp. 189-190.

[4]Jungherr, E.: The etiologic and diagnostic aspects of the fowl paralysis problem. J.A.V.M.A., lxxxvi (1935), pp. 424-432.

*The authors acknowledge the assistance of Dr. Erwin Jungherr in the examination and interpretation of histologic sections.

altered grossly in cases of osteopetrosis. Routine microscopic studies were not made on the parathyroids. Perivascular lesions, the exact significance of which has not been ascertained, have been noted in the central nervous system of several cases of osteopetrosis and in certain cases not manifesting osteopathy. Involvement of the peripheral nerves with typical gross lesions of neural lymphomatosis was observed in many cases. In all cases included in this report that have been studied microscopically, the gross diagnosis was verified.

Fig. 4—Cross-section of advanced osteopetrosis of tarsometatarsus — specimen B509A.

DISCUSSION

Evidence of the successful serial passage of strain 3 lymphomatosis-osteopetrosis gives support to its infectious and transmissible origin, either single or multiple. The intimate association of the lymphomatotic and osteopetrotic manifestations may be interpreted to suggest a possible single etiology. However, the occurrence of lymphomatosis in non-contact and contact control chickens and the virtual absence of osteopetrosis in the same controls may substantiate the opinion of Jungherr and Landauer,[1] based on morphologic criteria, that a complete identity of etiology seems unlikely.

In the six passages of the strain 3 agent, there was not apparent any diminution of the osteopetrotic propensity, although the condition was manifested in only two of four series of the third passage. The fact that the original donors and some of those of the subsequent passages did not show osteopetrosis suggests that the inciting factor or agent may remain latent.

Strain and probable family differences in the susceptibility of chickens to infection with the inoculum under study were observed. The range of difference was much greater in the occurrence of osteopetrosis than in lymphomatosis. Further work with highly inbred families of chickens may clarify the question of susceptibility or predisposition.

SUMMARY

Six serial passages were accomplished in chickens with the strain 3 agent, lymphomatosis - osteopetrosis. The first passage was initiated with the blood of three White Leghorn hens showing ocular and neural lymphomatosis. The osteopetrotic manifestations of strain 3, both gross and microscopic, resemble essentially those described for osteopetrosis gallinarum by Jungherr and Landauer.[1] For the purposes of this

paper, osteopetrosis has been designated and recorded separately from other expressions of lymphomatosis.

In the six passages, the combined incidence of the various manifestations of lymphomatosis and of osteopetrosis in 884 chickens inoculated at 1 to 22 days of age was 361 birds, or (40.8%). Osteopetrosis was detected in 80 birds, or (9%), of the inoculated group. Of those which developed lesions of lymphomatosis and/or osteopetrosis, 79 or (21.9%), showed gross and/or microscopic evidence of osteopetrosis. Among 775 uninoculated contact and non-contact control birds, 164 or (21.2%), showed lesions of lymphomatosis. With the possible exception of one clinical case still living, osteopetrosis did not occur in either control group. The post-inoculation observation period has not been completed in 159 birds of the fifth and sixth passage series.

The frequent occurrence of lymphomatosis and the virtual absence of osteopetrosis in the control groups may predicate epizoötiologic and/or etiologic differences.

Apparent differences in sex as well as strain and family susceptibility to osteopetrosis were observed. The incidence was significantly higher in males. In two of nine strains of White Leghorns, no manifestation of osteopetrosis were observed, while in one of the other strains, the morbidity was 12.7 per cent.

The Relation of Flies (Musca Domestica Linnaeus) to the Transmission of Bovine Mastitis*

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NUMEROUS STUDIES HAVE established the importance of the house fly (*Musca domestica Linnaeus*) in the dissemination of intestinal diseases. Graham-Smith[1] has shown that flies are important vectors in the transmission of various diseases. It has been demonstrated by Torry[2] that *Shigella dysenteriae, Eberthella typhosa,* and various Salmonellae can be carried by house flies. Yao, Yuan and Huie[3] have more recently studied the relation of flies, beverages and well water to gastrointestinal diseases in Peiping, China.

The literature concerning the relationship of the house fly to the specific etiologic agents of bovine mastitis is relatively meagre. Recently, however, Sanders[4] has reported that mastitis was experimentally transmitted to cows through the agency of the common house fly.

The purposes of this investigation on the relation of *M. domestica* to the transmission of bovine mastitis have been threefold: first, to determine the length of time that flies might carry *Streptococcus agalactiae;* second, to see if it were likely that the causative bacteria of mastitis could come into contact with flies feeding at the external surface of the teat sphincter; and third, to find if *M. domestica* trapped in a dairy barn, housing a herd with 46 per cent mastitis, carried the bacteria responsible for the disease. This last study was made during the summer months in a milking herd of 65 animals; 27 had streptococcus mastitis, 3 had staphylococcus mastitis, and 35 showed no mastitis according to the blood-agar plate method of diagnosis.

MATERIALS AND METHODS

Flies studied—M. domestica Linn., which had been propagated in the laboratory, were employed to determine the length of time they might carry *Str. agalactiae.*

Concerning the flies that were trapped in the dairy barn, no careful record was kept of

*From the Department of Bacteriology, University of Delaware.

[1]Graham-Smith, G. S.: Flies in relation to disease. Cambridge Univ. Press, London, 292 pp., (1913).

[2]Torrey, J. C.: Jour. Infect. Dis. 10: pp. 166-177, (1912).

[3]Yao, H. Y., Yuan, I. C., and Huie, D.: Natl. Med. Jour. China 15: pp. 410-418, (1929).

[4]Sanders, D. A.: Jour. A.V.M.A. xcvii: 120-123, (1940).

the number of flies that belonged to different genera. However, approximately 98 per cent of these flies were *M. domestica Linn.* and the remaining 2 per cent were *Stomoxys calcitrans Linn.* and *Haemotobia irritans Linn.* A bacteriological examination of the flies trapped in the dairy barn was limited to *M. domestica.*

Culture media employed.—For the isolation of bacteria from the flies, tryptose blood agar was used. The composition is given below:

Distilled water	1,000.0 ml.
Bacto-tryptose	20.0 Gm.
Glucose	2.0 Gm.
NaCl	2.5 Gm.
Na_2HPO_4	8.0 Gm.
Agar	20.0 Gm.

The above mixture was boiled and the reaction adjusted to pH 7.5. It was again boiled and filtered through absorbent cotton. Sterilization was carried out at 15 lb. pressure for 15 minutes. Citrated ox blood was added aseptically to the melted and tempered tryptose agar so as to give a final blood content of 7 per cent. Plates were then poured.

For the identification of the streptococci isolated from flies all differential culture media were prepared as recommended in the Manual of Methods for the Pure Culture Study of Bacteria.[5]

Analytical methods.—Coagulase activity and hemolysis, as recommended by Chapman et al.,[6] were employed in the determination of the pathogenicity of Staphylococci isolated from flies.

For the identification of *Str. agalactiae* colonies on the surface of tryptose blood-agar plates: If the agglutination occurred in the saline control, the serologic identification was made by the method of Coffey.[8] Kakavas[7] has developed a rapid agglutination method for identifying Lancefield Group B Streptococci. The details of this method will appear soon in the literature.

EXPERIMENT I.—The ability of laboratory-propagated *M. domestica* to carry *Str. agalactiae.*

Approximately 600 fly pupae were placed in a screened box, and while the flies were emerging they were fed sterile skim milk. When all the flies had emerged, and the pupae had been removed from the cage, the food consisted of skim milk containing *Str. agalactiae.* Fresh food was placed in the cage every 12 hours; a day's supply of milk was prepared each morning by adding 2 ml. of a young, actively growing broth culture of *Str. agalactiae* to approximately 100 ml. of sterile skim milk. By this procedure the milk upon which the flies fed each day contained enormous numbers of viable *Str. agalactiae.* In this manner, the flies were fed for 4 days. At the end of this time, the flies were fed on sterile skim milk for the duration of the study. At intervals an aliquot number of flies was removed from the cage through a wire cone. A sterile test tube containing 1 ml. of ether with absorbent cotton in the bottom was fastened to the cone and the flies were collected and killed in this container.

For the isolation of *Str. agalactiae* from the surface of the flies, individual examinations were made. Flies were placed in test tubes containing 3 ml. sterile saline and shaken vigorously for 7 minutes. Then the tubes were allowed to set for 15 minutes and 1 loopful of material was streaked over the surface of a tryptose blood-agar plate. The plates were incubated at 37 C. for 36 hours and then examined for colonies of *Str. agalactiae.*

The flies used for the above study were employed in an investigation of the bacteria contained internally. The following procedure was similar to that of Yao, Yuan and Huie.[9] The previously washed flies were pooled and placed in 70 per cent alcohol for 5 minutes and then washed 3 times in sterile saline. Following this treatment, varying numbers of flies were thoroughly ground in sterile mortars. Five ml. sterile saline were added to each mortar and allowed to set 5 minutes with occasional grinding. The bacteriologic examination of this material was made as described above.

The pure culture of *Str. agalactiae* employed in this study produced alpha prime colonies on the surface of tryptose blood-agar plates. When these colonies appeared on the surface of blood plates made from fly washings, they were quickly detected and presumptively identified by their alpha prime appearance. The identification was confirmed by a positive rapid agglutination test.

From the results shown in table I, it is seen that 6 per cent of the flies examined carried *Str. agalactiae* on their external surface for 7 days. After the flies had been fed on sterile milk for 2 days, all those examined showed the presence of *Str. agalactiae;* however, after 3 days, the percentage of flies carrying this organism externally, fell to 20 per cent.

RESULTS

The results showing the presence of *Str. agalactiae* inside the flies are only of relative value since fly pools were employed for this part of the study. However, it is

[5]Manual of methods for pure culture study of bacteria. Edited by the Committee on Bacteriological Technique of the Society of American Bacteriologists. Geneva, New York. (1938).

[6]Chapman, G. H., Berens, C., Peters, A., and Curcio, L.: Jour. Bact., xxviii, pp. 343-363, (1934).

[7]Kakavas, J. C.: Personal communication of unpublished data, (1940).

[8]Coffey, J. M.: Amer. Pub. Health Assoc. Year Book, (1937-1938).

TABLE I

The Duration of Time That *Streptococcus agalactiae* Is Carried by *Musca domestica Linn.*, Externally and Internally.

No. of Days Flies Fed on Sterile Milk	Presence of Str. Agalactiae on External Fly Washings			Presence of Str. Agalactiae in Internal Fly Washings			
	No. Flies Examined	No. Flies With Strep.	No. Flies Without Strep.	No. Fly Pools Examined	No. Flies Per Pool	No. Pools With Strep.	No. Pools Without Strep.
1	20	20	0	4	5	4	0
2	20	20	0	4	5	4	0
3	20	4	16	4	5	4	0
4	20	4	16	2	5	1	1
5	50	9	41				
6	50	7	43				
7	50	3	47	3	12 24 14	1 1 0	0 0 1
8	50	0	50				
9	50	0	50				
10	50	0	50	4	6 5 5 6	1 1 1 1	0 0 0 0
11	100	0	100				
14				4	10 10 10 10	0 0 0 0	1 1 1 1

shown that some of the flies examined internally carried *Str. agalactiae* for at least 10 days, while all the flies examined at the end of 14 days showed the absence of this microörganism in their internal washings.

EXPERIMENT II.—The availability of Str. *agalactiae* and pathogenic staphylococci at the external surface of the teat sphincter of mastitic cattle.

A study was made to determine if it were likely that flies might come in contact with the causative bacteria by feeding at the external surface of the teat sphincter of cows having mastitis. Seventeen infected cows in the barn were used in this investigation. Immediately before the morning milking period, a small sterile cotton swab was inserted into the teat canal of each quarter of these animals for a distance of approximately 3 mm. and removed. To be certain that the agents responsible for the mastitis were being shed in the milk at this time, a 25 ml. sample of milk was drawn aseptically from each quarter into a sterile bottle. The cows were milked by a stanchion machine and stripped by the same device. Immediately after each cow had been milked and stripped, the external surface of the teat sphincter was again swabbed to determine if the causative bacteria were present on this area following milking. The materials were taken to the laboratory and the cotton swabs and 1 loopful of each milk sample were streaked on the surface of tryptose blood-agar plates. The plates were incubated at 37 C. for 36 hours and examined for the presence of colonies of *Str. agalactiae* and hemolytic and double-zone colonies of staphylococci. Identification of *Str. agalactiae* was confirmed by the rapid agglutination plate method given previously. The staphylococcus colonies were picked into tubes of tryptose broth to obtain pure cultures. Their pathogenicity was determined by observing coagulase activity and hemolysis.

TABLE II

Presence of *Str. agalactiae* and *Pathogenic* Staphylococci at External Surface of Teat Shpincter Before and After Machine Milking and Stripping.

Cow	Qu.	Causative Bacteria Shed in Milk	Causative Bacteria Found at Teat Sphincter	
			Before Milking	After Milking
1	RR	— —	— —	— —
	RF	— —	— —	— —
	LR	— —	— —	— —
	LF	Pathogenic Staph.	Pathogenic Staph.	— —
2	RR	Str. agalactiae	Str. agalactiae	— —
	RF	Str. agalactiae	Str. agalactiae	— —
	LR	— —	— —	— —
	LF	— —	— —	— —
3	RR	— —	— —	— —
	RF	— —	— —	— —
	LR	Str. agalactiae	Str. agalactiae	— —
	LF	— —	— —	— —
4	RR	Str. agalactiae	Str. agalactiae	— —
	RF	Str. agalactiae	Str. agalactiae	— —
	LR	Str. agalactiae	Str. agalactiae	— —
	LF	— —	— —	— —
5	RR	— —	— —	— —
	RF	— —	— —	— —
	LR	Str. agalactiae	Str. agalactiae	— —
	LF	Str. agalactiae	Str. agalactiae	— —
6	RR	— —	— —	— —
	RF	— —	— —	— —
	LR	Str. agalactiae	Str. agalactiae	— —
	LF	— —	— —	— —
7	RR	(Blind)		
	RF	Pathogenic Staph.	— —	— —
	LR	Pathogenic Staph.	— —	— —
	LF	Pathogenic Staph.	Pathogenic Staph.	— —
8	RR	Str. agalactiae	Str. agalactiae	— —
	RF	Str. agalactiae	Str. agalactiae	— —
	LR	Str. agalactiae	Str. agalactiae	— —
	LF	Str. agalactiae	Str. agalactiae	— —
9	RR	Str. agalactiae	— —	— —
	RF	Str. agalactiae	Str. agalactiae	— —
	LR	Str. agalactiae	— —	— —
	LF	Str. agalactiae	Str. agalactiae	— —
10	RR	Str. agalactiae	Str. agalactiae	— —
	RF	Str. agalactiae	Str. agalactiae	— —
	LR	Str. agalactiae	Str. agalactiae	— —
	LF	Str. agalactiae	Str. agalactiae	— —
11	RR	— —	— —	— —
	RF	— —	— —	— —
	LR	— —	— —	— —
	LF	Pathogenic Staph.	Pathogenic Staph.	— —
12	RR	Str. agalactiae	Str. agalactiae	— —
	RF	Str. agalactiae	Str. agalactiae	— —
	LR	Str. agalactiae	Str. agalactiae	— —
	LF	Str. agalactiae	Str. agalactiae	— —
13	RR	Str. agalactiae	— —	— —
	RF	— —	— —	— —
	LR	Str. agalactiae	— —	— —
	LF	— —	— —	— —
14	RR	— —	— —	— —
	RF	Str. agalactiae	Str. agalactiae	— —
	LR	Str. agalactiae	Str. agalactiae	— —
	LF	Str. agalactiae	Str. agalactiae	— —
15	RR	— —	— —	— —
	RF	Str. agalactiae	Str. agalactiae	— —
	LR	Str. agalactiae	Str. agalactiae	— —
	LF	Str. agalactiae	Str. agalactiae	— —
16	RR	Str. agalactiae	Str. agalactiae	— —
	RF	Str. agalactiae	Str. agalactiae	— —
	LR	— —	— —	— —
	LF	— —	— —	— —
17	RR	— —	— —	— —
	RF	— —	— —	— —
	LR	Pathogenic Staph.	Pathogenic Staph.	— —
	LF	— —	— —	— —

RESULTS

Str. agalactiae and pathogenic staphylococci were not found on all external surfaces of teat sphincters of quarters which were shedding these bacteria in the milk. However, this was the exception rather than the rule when the examination was made immediately before a milking period. Of 41 quarters which were shedding *Str. agalactiae* or pathogenic staphylococci, 35 showed the presence of the bacterium responsible for the mastitis on the external surface of the teat sphincter.

Following the milking and stripping of the cows by machine, *Str. agalactiae* and pathogenic staphylococci were not found on any of the external surfaces of the teat sphincters that were examined.

It is evident from the results shown in table II that of the 17 mastitic animals which were shedding the specific etiological agents in the milk, 16 showed the presence of the infectious bacteria on at least one of the external surfaces of the teat sphincters immediately before milking.

EXPERIMENT III.—The occurrence of *Str. agalactiae* and pathogenic staphylococci in and on *M. domestica* which were trapped in a dairy barn housing mastitis cattle.

This study was made on a herd of dairy cattle of which 46 per cent were infected with mastitis. Twenty-seven cows had streptococcus mastitis, 3 had staphylococcus mastitis, and 35 cows were free of the infection. Only *M. domestica* were employed in this investigation.

Flies were trapped alive while the cows were present in the barn. This part of the investigation was made during the summer months. A conical hoop fly trap[9] was employed using molasses as bait. When a sufficient number of flies, usually 500, had accumulated in the trap, they were removed to the laboratory and killed with ether vapor. The external and internal washings of these flies were examined for the presence of *Str. agalactiae* and pathogenic staphylococci in the manner described previously. All hemolytic, partially-hemolytic, double-zone colonies and all colonies which appeared to be composed of streptococci were picked into tubes of tryptose broth and studied morphologically and physiologically. All cultures of staphylococci were identified as either pathogenic or nonpathogenic; no attempt was made to identify them as to species. All strep-

[9]Howard, L. O. and Bishopp, F. C.: U. S. Dept. Agr. Farmers' Bul. No. 1408, pp. 1-16, (1925).

tococcus cultures were identified as to species following the scheme of Sherman.[10]

TABLE III

Number of Flies Carrying Streptococci and Staphylococcic on Their External Surfaces.	
Total number of flies examined externally	2,629
No. of flies carrying Str. agalactiae.	0
No. of flies carrying pathogenic staphylococci	5
No. of flies carrying enterococci...	750
No. of flies carrying non-pathogenic staphylococci	526

RESULTS

The results in table III show that all the flies examined failed to carry *Str. agalactiae* on their external surface, while only a very few flies carried pathogenic stephylococci. Enterococci and nonpathogenic staphylococci were present on approximately 28 and 20 per cent, respectively, of the flies examined.

TABLE IV

Number of Flies Carrying Streptococci and Staphylococci Internally.	
Total No. of flies examined internally	1,100
No. of flies carrying Str. agalactiae.	0
No. of flies carrying pathogenic staphylococci	0
No. of flies carrying enterococci...	526

The flies that were examined did not harbor the causative bacteria within their bodies. Approximately 47 per cent of the flies showed the presence of enterococci in their internal washings.

DISCUSSION OF RESULTS

M. domestica carried *Str. agalactiae* externally and internally for a number of days after being fed on milk containing enormous numbers of the bacteria. With the exception of a few instances, flies feeding at external surfaces of teat sphincters of cows shedding the microörganisms would not ordinarily come in contact with so large a number of bacteria as was employed in the study dealing with the ability of flies to carry *Str. agalactiae.* The blood plates streaked with cotton swabs which had been placed in contact with the external surface of the teat sphincter previous to a milking period, never showed an abundance of colonies. In fact, the colonies of the specific etiologic agent usually numbered from 2 to 20 per plate. This indicated, in a rough fashion, that the causative bacteria were not numerous at the external surface of the teat sphincters examined.

The failure to find *Str. agalactiae* in or on any of the flies trapped in the dairy barn and the finding of very few flies that carried pathogenic staphylococci may seem rather surprising. However, after observing the habits of the flies in the barn, rarely a fly was found feeding at the teat orifice of an animal. The majority of flies were attracted to the grain and fodder fed to the cattle. Milking of the animals was conducted in a separate parlor and there was little or no milk present on the floor of the barn housing the cows. If milk containing viable specific etiologic agents of mastitis were present on the floor and bedding, the likelihood of the transmission of these bacteria to healthy cows would be increased greatly, both from a direct route and by the agency of flies. The employment of the fundamental principles of sanitary hygiene would greatly reduce this possibility.

During the summer months when the fly population is at its height, most cattle men keep the cows on pasture as much as possible and bring them to the barn for milking. The cows are usually not in the barn for any lengthy periods and the common house fly may not be in the cows' immediate environment for a sufficient length of time to become contaminated with the causative bacteria of mastitis. Another factor operating against the fly as an important agent in the transmission of mastitis is the relatively common use of fly spray in the dairy barn.

SUMMARY AND CONCLUSIONS

It is possible for flies (*M. domestica*) to carry *Str. agalactiae* externally and internally. *Str. agalactiae* and pathogenic staphylococci were available at the external surfaces of the teat sphincters in nearly all of the mastitic animals examined.

However, it seems very unlikely that the common house flies trapped in the dairy barn and studied in this investigation would have been important as agents for the transmission of bovine mastitis.

[10]Sherman, J. M.: Bact. Rev. 1: pp. 1-97, (1937). The author is now a member of the Sanitary Corps, U. S. Army.

The in vitro Neutralization of Hog-Cholera Virus with Hog-Cholera Antiserum*

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THE PHENOMENON OF *in vitro* neutralization of a virus by its homologous antiserum is utilized in the study of many virus diseases.

REVIEW OF LITERATURE

Infective and immunologic differences between some of the viruses and between strains of the same virus species can be demonstrated by neutralization technics. They have been used to advantage in the study of equine encephalomyelitis,[1, 2] vesicular stomatitis,[3] epidemic influenza,[4, 5] rabies,[6] yellow fever,[7] and St. Louis encephalitis.[8] To a limited extent and in a general way, neutralization technics have been employed in connection with certain studies on hog cholera.

In 1915, Craig[9] described experiments with mixtures of hog-cholera virus and hog-cholera antiserum. The amounts of the constituents in the mixture varied from 80 per cent virus and 20 per cent serum to 40 per cent virus and 60 per cent serum. The mixtures were heated in a water bath, but he did not state the temperature or length of time incubated. Susceptible pigs, each weighing about 50 lb., were injected with 1 to 4 cc. of a mixture. Subsequently, they were subjected to cholera by either pen exposure or direct inoculation with virus. This resulted in the death from cholera of all but one. Healy and Gott[10] reported mixing together 3 cc. of virus and 20 cc. of antiserum and incubating at 37 C. for 48 hours. The mixture was then injected into two healthy pigs, each weighing approximately 100 lb. For two weeks following the injection, both animals remained well. They were then placed in a pen from which cholera-sick pigs had been removed. They remained in this environment for two weeks without developing cholera, although one of them showed a thermic reaction beginning the third day and continuing for five days. Both pigs were later hyperimmunized for the production of antiserum.

The 1929 and 1930 reports of the chief of the Bureau of Animal Industry, U. S. Department of Agriculture, contain summarized reports of work done in the Biochemic Division with mixtures of hog-cholera virus and antiserum. Virus-serum mixtures in the ratio of 1 : 10 were prepared and portions inoculated into susceptible pigs. The quantity injected was 1 cc. per dose, subcutaneously. Some of the pigs received one dose, others two doses, and still others three doses. An interval of a few days elapsed between the treatments of those which received two and three doses. It is reported that the test pigs neither became sick nor were they effectively immunized as a consequence of the treatment. It was assumed that the quantity of virus contained in each dose (0.1 cc.) was sufficient to have induced the disease, since the controls received 0.1 cc. of the untreated virus and all sickened and died from cholera. The objective in all these experiments was chiefly that of developing an "attenuated virus vaccine" suitable for use as a protective treatment against hog cholera.

MATERIALS AND METHODS

The method of conducting the neutralization tests was simple and expedient. Measured quantities of the defibrinated blood of cholera-sick swine (the virus) were mixed with measured quantities of hog-cholera antiserum (the serum). The proportion of virus to serum in the different mixtures was varied. The ratio ranged from 1 : 0.67 to 1 : 30. The mixtures were thoroughly agitated and then placed in a water bath at 37.5 C. for different lengths of time. The incubation time on one series was one hour, and five hours for a second series. Those held for five hours were agitated twice during the incubation period. Immediately following the incubation period, portions of the mixtures were injected into susceptible swine. The amount was adjusted to the body weight and the injection made into the muscles on the medial surface of the thigh.

Virus.—The virus used in all tests was virulent and potent. Proof of its pathogenicity had been determined. The susceptible pigs injected with it developed symptoms and presented blood pictures and postmortem changes characteristic of cholera.

It is unfortunate that we could not work with a quantity of virus representing its m.l.d. A definite and specific m.l.d. for the virus of this disease has not been established. The minimal fatal doses of hog-cholera virus reported in the literature vary greatly. King and Wilson[11] reported as the minimal fatal dose a

*Paper No. 1918, scientific journal series, Minnesota Agricultural Experiment Station; contribution from the Division of Veterinary Medicine. Presented before the Section on Research at the seventy-eighth annual meeting of the American Veterinary Medical Association, Indianapolis, Ind., August 11-15, 1941.

quantity of virus somewhere between 1/215 and 1/300 cc. Roderick and Schalk[12] found that 1/50,000 cc. of virus was sufficient to cause fatal cholera in 30- to 60-lb. pigs. According to McBryde,[13] a dilution equal to 1/300,000 cc. produced the disease in pigs weighing 40 to 60 lb. Whiting,[14] working along similar lines, found that the period of incubation and the course of the disease were shorter in pigs receiving 1 cc. of virus than in those receiving one half of that quantity.

The differences just cited are wide. They suggest the possibility of differences in virulence and potency of the virus as well as differences in the susceptibility of the pigs tested. For these reasons, it was considered advisable to use a quantity of virus that was certain to infect. The quantity used more nearly approximated that administered when applying the serum and virus treatment as a preventive procedure on pigs of the size and weight employed. The specific amount of the virus injected varied with the body weight, but was given at the rate of 2.5 cc./50 lb. Thus, should the pigs fail to sicken and develop cholera subsequent to receiving the virus-serum mixtures, it would be evidence that the virus was neutralized by the serum.

Antiserum.—The antiserum employed in these studies was the regular anti-hog-cholera serum, such as is used in the field where serum and virus treatments are administered. It was the product of a commercial establishment preparing antiserum and virus under license by the federal government. The quantity of serum was adjusted to the body weight; it varied between 1.66 cc./50 lb. and 75 cc./50 lb. In some cases, the amounts were below those generally recommended and used in connection with the serum and virus treatment, whereas in other cases the amount was much greater.

RESULTS

Tables I and II summarize the results obtained with the pigs injected with the virus-serum mixtures, which is the only way we have of noting the effects of the *in vitro* manipulations with these substances. When the proportion of virus was in excess of serum (1:0.67—pigs 10778, table I, 10842 and 10843, table II), the infectivity of the virus was not destroyed. It made no difference whether the mixture was incubated for one hour or for five hours. In fact, its virulence seems not to have been reduced because two of the pigs sickened and died from cholera in 9 days and the third in 13 days.

For the ratios of virus to serum of 1:1.34 and 1:2.5, with incubation periods of one and five hours, the results were quite different. The pigs receiving the mixtures incubated for the shorter period all sickened and died from cholera in from 9 to 17 days after injection. All of those receiving the virus-serum mixtures of similar proportions, but incubated for five hours, lived. This difference, we believe, was due to the neutralization of the virus by the antiserum. We feel certain that the virus was not destroyed by the longer incubation time, in view of the fact that its virulence was unimpaired when held for a similar length of time in connection with the tests with a virus-serum ratio of 1:0.67, reported above. Furthermore, the results of several investigations directed to the effects of various temperatures and time on the potency or virulence of hog-cholera virus indicate that the virus is not destroyed if held at 37 C. for five hours. Dorset and McBryde[15] reported holding virulent hog-cholera blood at 60 C. for 30 minutes without destroying its infectiveness. McArthur[16] heated virus at 60 C. for four hours and to 65 C. for two hours and still it proved virulent when injected into susceptible swine. More recently, Ray and Whipple[17] described heating phenolized (0.5%) hog-cholera virus to 36-37 C. for 132 hours without influencing its lethal properties. They also reported the virulence of the virus uninfluenced when held at 44.5 C. for ten hours.

Since the difference between the two groups was marked, *i.e.*, between virulence

TABLE I — *In vitro* Neutralization Tests with Hog-Cholera Virus and Hog-Cholera Antiserum (Mixtures Heated to 37.5 C. for One Hour)

Pig No.	Virus-Serum Ratio	Results*
10778	1 : 0.67	Died in 13 days
10776	1 : 1.34	Died in 14 days
10840	1 : 1.34	Died in 9 days
10774	1 : 2.5	Died in 17 days
10884	1 : 2.5	Died in 9 days
10777	1 : 5	Survived—133 days later challenged with virus—survived
10775	1 : 10	Survived—133 days later challenged with virus—survived

*All deaths were due to hog cholera.

TABLE II—*In vitro* Neutralization Tests with Hog-Cholera Virus and Hog-Cholera Antiserum (Mixtures Heated to 37.5 C. for Five Hours)

PIG NO.	VIRUS-SERUM RATIO	RESULTS*
10842	1 : 0.67	Died in 13 days
10843	1 : 0.67	Died in 9 days
10798	1 : 1.34	Survived— 84 days later challenged with virus—Died in 7 days
10799	1 : 1.34	Survived— 84 days later challenged with virus—Survived
10808	1 : 1.34	Survived— 84 days later challenged with virus—Died in 9 days
10809	1 : 1.34	Survived— 84 days later challenged with virus—Died in 9 days
10810	1 : 1.34	Survived— 84 days later challenged with virus—Died in 9 days
10791	1 : 2.5	Survived— 84 days later challenged with virus—Died in 6 days
10793	1 : 2.5	Survived— 84 days later challenged with virus—Died in 5 days
10794	1 : 2.5	Survived— 84 days later challenged with virus—Died in 8 days
10795	1 : 2.5	Survived— 84 days later challenged with virus—Died in 7 days
10805	1 : 2.5	Survived— 84 days later challenged with virus—Survived
10797	1 : 5	Survived— 84 days later challenged with virus—Died in 5 days
10801	1 : 5	Survived— 84 days later challenged with virus—Survived
10803	1 : 5	Survived— 84 days later challenged with virus—Died in 6 days
10806	1 : 5	Survived— 84 days later challenged with virus—Died in 9 days
10807	1 : 5	Survived— 84 days later challenged with virus—Died in 7 days
10780	1 : 10	Survived— 84 days later challenged with virus—Died in 11 days
10792	1 : 10	Survived— 84 days later challenged with virus—Died in 4 days
10796	1 : 10	Survived— 84 days later challenged with virus—Survived
10800	1 : 10	Survived— 84 days later challenged with virus—Died in 23 days
10802	1 : 10	Survived— 84 days later challenged with virus—Survived
10804	1 : 10	Survived— 84 days later challenged with virus—Died in 7 days
10781	1 : 20	Survived—116 days later challenged with virus—Died in 13 days
10782	1 : 30	Survived—116 days later challenged with virus—Died in 13 days

*All deaths were due to hog cholera.

and avirulence, and since it was not a matter of the virus being killed by heat, it appears that the longer incubation period afforded a greater opportunity for a more complete combination or union to occur between the virus and antibody so that its infective property was inhibited or destroyed. Proof of the avirulence of the virus in these mixtures is shown by the fact that when the susceptibility of the pigs was challenged, most of them succumbed to cholera. The challenge consisted of an intermuscular injection of virulent cholera virus. An interval of 84 days elapsed betweeen the administration of the virus-serum mixtures and the challenging dose of virus. Eight of the ten pigs in this group sickened and died from cholera and two remained well. The question, of course, is, were these two pigs immune before receiving the virus-serum injections or did they become immune as a consequence of the injection. While this can not be answered with assurance, we believe that they were not immune at the start. If they developed an immunity as a consequence of the virus-serum mixtures, it would seem that some of the antibody was removed within the body of the pig, leaving sufficient virus to stimulate the production of immune substance (antibodies).

All of the pigs injected with the virus-serum mixtures at ratios of 1:5 and 1:10 remained well. After an interval of 84 days in some cases and 133 days in others, they were exposed to hog cholera by direct inoculation of virus. Two pigs received mixtures that had been incubated for one hour and eleven received mixtures that had been incubated for five hours. Both of the former and three of the latter survived the challenging dose of virus.

One pig received a virus-serum mixture in the ratio of 1:20 that was incubated for five hours, and another pig received a mixture of 1:30 that had been incubated for a similar length of time. Both remained well as a result of the treatment, but when challenged 116 days later, both succumbed to cholera.

SUMMARY AND CONCLUSIONS

Virulent hog-cholera blood and hog-cholera antiserum were mixed together in

various proportions and incubated at 37.5 C. for different lengths of time. The ratios of virus to antiserum in the mixtures ranged from 1:0.67 to 1:30. One series of such mixtures was incubated for one hour and a second series for five hours. The mixtures were so adjusted that the amount of virus each pig received was 2.5 cc./50 lb. This amount is much in excess of what might be considered an m.l.d., but was chosen for the purpose of insuring an adequate dose.

The virulence or infectiveness of the virus in the virus-serum mixtures in a ratio of 1 : 2.5 or less was not neutralized when incubated for one hour. The pigs receiving such mixtures promptly sickened and died from hog cholera. The mixtures of 1:5 and 1:10 were neutralized. When the incubation time was five hours, the virus was neutralized in the mixture of the ratio of 1:1.34 and all of the larger ratios. This appears to be due to a more complete combination or union between the virus and serum rather than a "heat-killed" virus, since all of the pigs injected with 1 : 0.67 mixtures heated for the same length of time sickened and died from cholera.

All pigs which survived the virus-serum inoculations were subsequently challenged by injections of virulent virus with the result that 72 per cent sickened and died from cholera and 28 per cent developed an immunity.

The results show that an *in vitro* neutralization of hog-cholera virus by hog-cholera antiserum does occur. When mixtures of virus and serum were incubated for one hour at 37.5 C., it required approximately five times as much antiserum as virus to effect a neutralization, whereas when incubated for a longer period (five hours), about an equal amount of antiserum and virus resulted in neutralization.

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Incidence of Gastrointestinal Nematodes of Cattle in the Southeastern United States*

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ALTHOUGH SOME REPORTS are available on the species of nematodes found in the gastrointestinal tract of cattle in the United States, there are no data on the comparative incidence or intensity of infection with the various species, except for scattered articles on outbreaks of verminous gastroenteritis in the bovine species.

Since 1938, when investigations on internal parasites of cattle were begun in this laboratory, examinations have been made to determine the relative incidence of the species of nematodes. At the present time, data are available from partial or complete examinations of the abomasum, small intestine, and large intestine of 84 cattle ranging in age from 1 to 18 months. Because the animals examined were from a variety of sources, the numbers in any individual age or breed group or from any one farm are too small for critical analysis of the effects of any factors on the worm burdens. However, in view of the facts that information of this type is slow in accumulating and that the present number of examinations is insufficient to give an idea of the relative parasite incidence, the data thus far obtained appear to be worth reporting.

MATERIALS AND METHODS

In the search for parasites, the animals were eviscerated as soon as possible after death, and the parts of the abdominal viscera to be examined were transported to the laboratory. To prevent the worms from spilling from one location to another while handling the viscera, ligatures were placed at the junctions of the fourth stomach with the omasum and the small intestine, and at the ileocecal valve. In the laboratory, the stomach and the small intestine were opened separately in water and the mucosa was thoroughly washed to remove any parasites present. Six of the 80 stomachs examined were only superficially washed to remove Haemonchus spp. and were not considered as having been examined for *Ostertagia ostertagi* and *Trichostrongylus axei*, small worms that are usually so embedded in the mucosa that they can be removed only by vigorous washing.

After the volume was recorded, the contents and washings from these organs were stirred to obtain an even suspension of parasites in the fluid and a fraction sample; usually, one-fortieth of the total was taken from each mixture. The total number of worms of each species of small nematodes present was then estimated from the number in the samples. The numbers of larger worms, namely, adult stomach worms (Haemonchus spp.) and hookworms (*Bunostomum phlebotomum*), were determined by screening the entire contents of the stomach and small intestine. The washings also were searched for specimens of the smaller species that may have been present in numbers too small to be detected by sampling. Nodular worms (*Oesophagostomum radiatum*) and whipworms (Trichuris spp.) were counted by screening the contents of the large intestine. Total counts rather than estimations were made for the large stomach worms, hookworms, nodular worms, and trichurids, except in four instances where it was impossible to bring the viscera to the laboratory. In these instances, large numbers of these parasites were present. Therefore, samples of the contents and washings were collected, preserved and brought to the laboratory for examination and estimation of the numbers present.

The origin of the cattle examined was as follows: Auburn, Ala., and vicinity, 41; Selma, Ala., and vicinity, 16; Kissimmee, Fla., 2; Gainesville, Fla., 2; Athens, Ga., 5; Moultrie, Ga., 6; Starkeville, Miss., 6; and Baton Rouge, La., 6. Forty-five of the subjects slaughtered were in good condition and fit for food.

Five of the animals from Moultrie, Ga., were untreated yearlings examined in connection with tests with phenothiazine conducted in the fall of 1939 (Swanson, Porter, and Connelly[1]). In view of the fact that the contents of the stomach and the small intestine were not sampled before screening, the numbers of Ostertagia, Cooperia, and Strongyloides were not determined in two of these yearlings. The sixth animal, a 3-month-

*From the Regional Animal Disease Research Laboratory, Bureau of Animal Industry, U. S. Department of Agriculture, Auburn, Ala.

[1]Swanson, L. E.: A note on the parasitic fauna of Georgia. Proc. Helminth. Soc. Washington, viii (1941), pp. 62-63.

TABLE I—The Gastrointestinal Nematodes of Cattle Arranged According to the Percentage Incidence

PARASITE	LOCATION	NO. ANIMALS EXAMINED*	PERCENTAGE PARASITIZED	RANGE IN NO. OF PARASITES
Cooperia punctata	Small intestine	81	91	2-129,360
Haemonchus contortus	Abomasum	80	83	6-10,500†
Ostertagia ostertagi	Abomasum	74	74	5-4,000
Bunostomum phlebotomum	Small intestine‡	81	62	1-2,216
Oesophagostomum radiatum	Large intestine	78	59	1-6,256
Trichostrongylus axei	Abomasum	74	47	2-28,000
Trichuris spp.§	Large intestine	78	44	1-372
Cooperia pectinata	Small intestine	81	32	4-27,668
Strongyloides papillosus	Small intestine	81	21	6-2,600
Haemonchus similis	Abomasum	80	18	†
Nematodirus helvetianus	Small intestine	81	15	2-4,500
Cooperia oncophora	Small intestine	81	11	19-258
Trichostrongylus colubriformis	Small intestine	81	9	100-9,700
Capillaria longipes	Small intestine	81	7	20-400

*Complete examination not made on all 84 cattle.
†*H. contortus* and *H. similis* counted together.
‡Specimens also attached to abomasum in 8 cases.

old calf from the same locality, was examined, in coöperation with Dr. L. E. Swanson, at the same time.

The remainder were examined in an attempt to diagnose the cause of losses or poor condition in various herds. Several of the cases, especially those in which extensive parasitism was suspected, were referred to the writer by the Veterinary Clinic of the Alabama Polytechnic Institute.

OBSERVATIONS AND DATA

Although this paper deals with the gastrointestinal nematodes parasitic in the bovine species, it is pertinent to note that tapeworms (Moniezia sp.) were encountered in 14 of 81 animals in which the small intestine was examined. One 4-month-old calf had 22 specimens and one yearling had 20 specimens. The remainder, including a month-old calf which harbored 3 immature specimens, had from 1 to 3 worms each.

The nematodes represented 10 genera and 15 species. The nematode parasites are arranged, in the order of their incidence, in table I and are discussed in the order of their location, beginning with those in the abomasum and continuing with species in the small intestine and large intestine.

ABOMASUM

Haemonchus contortus (Rudolphi, 1803) was the second most common parasite encountered and was present in the abomasum of 83 per cent of 80 animals examined from all areas. Eight of the 14 animals not infected with this parasite were among 20 calves under 6 months of age. The number of stomach worms found, including *H. similis*, ranged from 6 in a 2-month-old calf to 10,500 in a yearling steer (table I).

H. similis (Travassos, 1914), previously reported from Louisiana (Dikmans[2]) and Georgia (Swanson, Porter, and Connelly[1]; Swanson[3]), was encountered in this survey in cattle in the states named as well as in those from Alabama and Florida. This species was not encountered in 6 yearlings from Starkeville, Miss. Eighteen per cent of 80 cattle harbored the parasite and, with one exception, it occurred when H. *contortus* was also present.

Ost. ostertagi (Stiles 1892), was found in small to moderate numbers in 74 per cent of 74 animals representing all areas. Twenty mature specimens were found in one calf 34 days old and the largest number found was 4,000, taken from a yearling steer slaughtered at Baton Rouge, La.

Trichostrongylus axei (Cobbold, 1879), was found in 47 per cent of the animals from all areas. With two exceptions, this parasite occurred in fairly small numbers, 50 per cent of

[2]Dikmans, G.: Second preliminary report on parasites found in ruminants at the municipal abattoir, Baton Rouge, La. Louisiana Exp. Sta. Bul. 186 (1923).

[3]Swanson, L. E., Porter, D. A., and Connelly, J. W.: Efficacy of nonconditioned phenothiazine in removing worms from the alimentary tract of cattle. J.A.V.M.A., xcvi (1940), pp. 704-707.

the infected animals having less than 500. Two yearling steers harbored approximately 28,000 and 19,000, respectively, the largest numbers of *T. axei* recorded in the entire series.

SMALL INTESTINE

T. colubriformis (Giles, 1892) is apparently uncommon in southern cattle. It was found in the upper small intestine of 7 of 81 animals (9 per cent) examined. Six of the infected animals were from the vicinity of Auburn, Ala. Five of them were calves ranging from 1 to 6 months of age from one dairy and harbored from 100 to 750 specimens; the sixth, a yearling from a neighboring farm, harbored 9,700 specimens. The seventh to have this parasite was a yearling from Athens, Ga., from which 200 specimens were recovered.

Cooperia punctata (von Linstow, 1907) was the most prevalent of any of the nematodes, occurring in 74 of 81 animals (91 per cent) examined from all areas. The parasite occurred in calves of all ages, over 3,000 being found on one occasion in a month-old calf. The highest estimated numbers seen were 129,360, collected from a yearling, and 113,000, collected from a 5-month-old calf. The numbers recovered from all infected animals ranged from 25 to 129,360.

C. pectinata (Ransom, 1907) was found in 26 of 81 animals (32 per cent) examined. Forty-five per cent of the infected animals had less than 500 worms each. The largest number (27,668) was found in a yearling in which there also were 110,672 *C. punctata*. According to Dikmans,[4] this species has been collected from cattle in Texas, Louisiana, Mississippi, Georgia, and Florida, but was not found in examinations of cattle from Virginia and Maryland. It was encountered in all of the states represented in this survey.

C. oncophora (Railliet, 1896) was found in only 9 animals (11 per cent), ranging in age from 6 months to 1 year. Of the infected animals, 3 were from Athens, Ga.; 4 from the vicinity of Selma, Ala.; and 2 from the vicinity of Baton Rouge, La. The numbers found were 100, 200, 80, 100, 142, 19, 48, 258, and 150, respectively. Judging from the low incidence and intensity of infection as well as from previous records, it is evident that this species is comparatively rare in the South. Price[5] observed this species in sheep, but did not include it in a list of parasites encountered in Texas cattle. Dikmans[2] included *C. oncophora* in a list of parasites recovered from cattle at Baton Rouge, La., but in a later paper[4] he stated, "In a fairly large number of cattle examined in Louisiana and Texas, the writer encountered large numbers of *C. punctata* and *C. pectinata* but failed consistently to find *C. oncophora*."

Bunostomum phlebotomum (Railliet, 1900) was found in small numbers in all areas where cattle were examined. Fifty of 81 cattle (62 per cent) were positive. In eight instances, hookworms were attached to the wall of the abomasum as well as to that of the upper small intestine. Fifty per cent of the infected animals harbored less than 25 worms each while 30 per cent harbored 25 to 200 specimens each. The largest numbers found were 2,216 in an 18-month-old steer at Gainesville, Fla., and 1,374 in a 3-month-old calf at Moultrie, Ga.

Strongyloides papillosus (Wedl., 1856) was present in 17 of 81 animals (21 per cent). Three of the infected animals were yearlings and the remainder were 1 to 6 months old. Usually, small numbers of worms (12 to 500) were found; the largest number (2,600) occurred in a 4-month-old calf.

Nematodirus helvetianus (May, 1920) was encountered in 12 of 81 animals (15 per cent). The infected animals came from eastern Alabama and northern Georgia. With three exceptions, 2 to 400 specimens were recovered. Two 4-month-old calves harbored approximately 2,000 to 3,000 specimens, respectively, in addition to 1,000 *C. punctata* each. Stomachs were not examined. The third calf, also 4 months old, harbored 4,500 *N. helvetianus* in addition to a small number (less than 100) of *Ost. ostertagi* and approximately 800 *C. punctata*.

Capillaria longipes (Ransom, 1911) was recovered from the small intestine of 6 out of 81 cattle. Two 4-month-old calves and a yearling from near Auburn, Ala., and 3 yearlings from Starkeville, Miss., were infected. The calves harbored 50 and 360 specimens, the yearlings, 20, 25, 33, and 400 specimens, respectively. Considering the size of individual infections and the low incidence, it appears that this species is relatively unimportant.

LARGE INTESTINE

Oesophagostomum radiatum (Rudolphi, 1803) was found in 46 of 78 animals (59 per cent). The numbers varied from 1 in a 2-month-old calf to 6,256 in a yearling steer (Porter, Simms, and Cauthen[6]). Fifty per cent of all animals in which this parasite was recorded had less than 50 specimens each.

Trichuris spp. usually were found in the cecum and were most numerous in the region of the ileocecal valve. In view of the fact that protrusion of the spicules or spicule sheaths of the males was seen only in occasional specimens, specific diagnosis was not made in all cases. Diagnosis of *T. ovis* (Abildgaard, 1795) was made frequently, and specimens identified as *T. discolor* (von Linstow, 1906) were found in 6 of the 34 animals harboring this genus. From 1 to 372

[4]Dikmans, G.: Parasitic enteritis in calves. Vet. Med., xxxiv (1939), pp. 28-29.

[5]Price, E. W.: Proc. Helminth. Soc. Washington, 107th meeting. J. Parasitol., xiv (1928), pp. 201-202.

[6]Porter, D. A., Simms, B. T., and Cauthen, G. E.: Field tests with phenothiazine as an anthelmintic in cattle. J.A.V.M.A., xcix (1941), pp. 272-278.

whipworms were encountered. Four dairy calves, 4 to 6 months of age, harbored 160, 191, 372, and 210 whipworms, respectively, or approximately 70 per cent of the total number encountered.

DISCUSSION

All of the species of gastrointestinal nematodes collected from cattle in this survey were reported previously in the United States (Ransom, 1911, Dikmans,[2] Price,[5] Stoll,[7] and others). With few exceptions, these species were collected in the South. Apparently, however, this is the first report of *T. discolor* and *N. helvetianus* in the Southeastern States.

The data presented here also show that the nematodes occurring most frequently are *C. punctata*, a trichostrongyle of the small intestine; *H. contortus*, the common stomach worm; *Ost. ostertagi*, a small trichostrongyle occurring in the abomasum; *B. phlebotomum*, the cattle hookworm; and *Oes. radiatum*, the cattle nodular worm. These known pathogens or closely related ones also have been encountered frequently in quantitative surveys in widely separated parts of the world. In Australia (Roberts[8]), *H. contortus* (87%), *Oes. radiatum* (76%), *Ost. ostertagi* (75%), and *T. axei* (75%) were among the first five in incidence. *C. pectinata* was second (78%), *C. punctata sixth* (72%), and *B. phlebotomum* eighth (45%) in the order of occurrence. In Puerto Rico (Andrews and Maldonado[9]), the species of gastrointestinal nematodes most frequently encountered were: *B. phlebotomum* (77%), *Oes. radiatum* (64%), *C. punctata* (55%), *H. contortus* (50%), *Trichuris ovis* (41%), and *H. similis* (22%). *Ost. ostertagi* followed in seventh place (18%) and *T. axei* was ninth (14%).

Due to differences in climate, animal husbandry practices, proportion of different-aged animals, number of animals examined from the same premises, and other factors, it is to be expected that some variation in incidence will be found in widely separated areas. The relatively low incidence of *Ost. ostertagi* and *T. axei* in Puerto Rico and the relatively high incidence of *C. pectinata* in Australia in comparison with the findings reported here are particularly noticeable. According to Dikmans,[4] the latter species appears to be more prevalent than *C. oncophora* in the southern United States, with the reverse being true in the North. The relative prevalence of these species in the present study seems to bear out the fact that *C. pectinata* is more prevalent than *C. oncophora* in the South.

The opportunities afforded in various veterinary laboratories in this country should be a challenge to investigators to record the occurrence and prevalence of the various species of internal parasites. Only by the accumulation of such data can their distribution and relative importance be determined.

SUMMARY

Partial or complete examinations were made of the abomasum, small intestine, and large intestine of 84 cattle, ranging in age from 1 to 18 months, from Alabama, Georgia, Florida, Mississippi, and Louisiana. In some instances, only the stomach, in others only the intestine was examined. Fifteen species of nematodes representing ten different genera were recovered. The numbers of specimens were counted or estimated in most cases.

The percentage incidence of the various species was: *Cooperia punctata*, 91 per cent; *Haemonchus contortus*, 83 per cent; *Ostertagia ostertagi*, 74 per cent; *Bunostomum phlebotomum*, 62 per cent; *Oesophagostomum radiatum*, 59 per cent; *Trichostrongylus axei*, 47 per cent; Trichuris spp. (*T. ovis*, *T. discolor*), 44 per cent; *Cooperia pectinata*, 32 per cent; *Strongyloides papillosus*, 21 per cent; *Haemonchus similis*, 18 per cent; *Nematodirus helvetianus*, 15

[7]Stoll, N. R.: Observations on cattle nematode infections with a demonstration of their secondary transmission to grazing sheep. J. Parasitol., xxii (1936), pp. 386-407.

[8]Roberts, F. H. S.: The occurrence and prevalence of gastro-intestinal helminths in apparently healthy cattle in Queensland, Australia. J. Comp. Path. & Therap., lii (1939), pp. 160-165.

[9]Andrews, J. S., and Maldonado, J. F.: A preliminary note on the internal parasites of Puerto Rican cattle with special reference to those species found in calves suffering from "tropical diarrhea." J. Agr. Univ. Puerto Rico, xxiv (1940), pp. 121-132.

Reindeer Compared with Domestic Animals in Relation to Fattening, Sex, and Increase*

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THE RECENT comprehensive article by Hirzel[1] on the proportions of muscle, fat, and bone in mutton and beef, in which papers by several workers in America are cited, has caused me to make some comparisons on the growth and fattening of reindeer. I realize that the food given to animals is the most important factor in the fattening process, but sex also may affect the rapidity of gains. It is well known that castrated animals are more lethargic than entire beasts and, under certain conditions, they make slower gains.

In order to show what a marked effect sex has on reindeer, I will mention the visible effects of castration. For instance, if a reindeer buck is castrated in July when the antlers are in the velvet, they will remain attached until the following spring and the velvet will not be scraped off. Normally, a reindeer buck sheds his antlers after the rut in November. If the buck is castrated late in August after the velvet has been shed, the shock of the operation causes the antlers to fall off in a few days. In reindeer, the antlers are well named secondary sex characters and are more closely linked to sex than the horns of cattle or sheep. Nevertheless, the comparisons which follow will show how important a stimulus the hormones of sex are to reindeer.

Eskimo herders assisted in this work. The Eskimos know what meat is fattest and, therefore, what is best to eat at different times of the year. I found in Lapland that bucks are killed in the summer for sale, as they are known to be the fattest animals in the herds.

The castration of reindeer by the Lapps was not done for the purpose of improving the palatability of the meat or to make the animals fatter, but to prevent the loss of flesh during the rutting season. Therefore, the animals were castrated shortly before it, with a view of marketing them later in the year.

GROWTH IN RELATION TO SEX

In mammals, the males are by nature larger than the females, but in the domesticated animals, such as cattle and sheep, the steers and wethers differ from their parents by growing larger.

Hirzel[1] states: "The rates at which breeds mature go hand in hand with the food supply." Hammond (quoted by Hirzel) says, "The size of the animal is dependent on its environment." These quotations refer in a general way to the growth of the body disregarding sex. Another quotation given by Hirzel (from Hankins, 1932) is: "Sex differences in carcass proportions will only become marked as sexual maturity is approached in the heifer; but more evidence is necessary before concluding what exactly these differences are, and to what extent steers and heifers differ at various ages. The ratio of fat to lean in steers and heifers remains the same up to approximately 625 lb. live weight and thence rises in favour of the heifer. Steers and heifers have the same fat to bone ratio under 15 months."

In commenting on these facts given by Hirzel, on the more rapid gains of heifers

*From the Ontario Research Foundation. Permission to publish this report was granted by Mr. R. A. Gibson of the Department of Interior, Ottawa. The dates of fawning of yearling reindeer were furnished by Mr. S. Mason.

[1]Hirzel, R.: Factors affecting quality in mutton and beef with special reference to the proportions of muscle, fat and bone. Onderstepoort J. Vet. Sci., xii (1939).

(*Continued from preceding page*)

per cent; *Cooperia oncophora*, 11 per cent; *Trichostrongylus colubriformis*, 9 per cent; *Capillaria longipes*, 7 per cent.

Tapeworms (Moniezia sp.) also were encountered in 14 of 81 animals in which the small intestine was examined.

over steers, I would suggest that if comparison had been made with bulls of the same age, there is a probability that the gains might have been the same. The heifers being of the right age may have been affected by hormones, and the steers lacked this stimulation. I make this statement knowing that in cattle the normal mating season has been changed even in some of the beef herds, and they can not be compared with species like sheep, which mate at the proper season, in which the growth of the uncastrated lambs of both sexes should harmonize. The best proof that I can offer is what I have learned about reindeer. In my notes, I mention the antlers because they are an outward indication of sex development.

Every year, the bulls on the same range under similar conditions outstrip the steers, getting fat long before them. This is in preparation for the mating season, and they succeed in so doing despite the fact that they were run down the previous autumn following the rut. The steers in the herd lose condition after December and do not regain their optimum weight until the following autumn. I attribute the slower gain in the steers to their more indolent nature, especially when, owing to bad weather, they have to work for their food. Whether this is the right explanation or whether it is the lack of hormones, I am not certain; probably it is a combination of both factors. In this connection, it should be mentioned that the sexes in reindeer and caribou naturally separate at fawning time. This separation is complete until August, when the bucks rejoin the does.

ANNUAL FATTENING OF REINDEER AND ITS RELATION TO SEX

Bucks.—These are the first to fatten in the spring. Toward the end of March, their condition already shows improvement. The antlers are growing rapidly and in June these animals are fat and sleek coated, though the fat is still reddish and vascular. They reach the peak of condition in August, when the back fat becomes hard and white. The velvet is now scraped off the antlers, just before the rut begins on the first days in September. The meat of bucks killed before the mating season is tender and free from any sex odor. After the first of September, the neck swells and other signs of sexual activity are manifest. The antlers are round and the tines long and sharp-ended. Bucks shed their antlers after the rut is over early in November; at this time they are thin and gaunt.

Does.—The does are in good condition in the spring until the fawns are born, *i.e.*, from April 10 to about May 15, when most of the fawns have been dropped. From this time onward, they nurse their fawns and lose flesh, though only a few become actually thin. After the pasture improves, the majority show no signs of strain. The does drop their antlers a few days after the fawn is born. New antlers start growing and are full sized before September 1, when the velvet is peeled off. The does do not reach their peak of condition until October—after the fawn has been weaned and the rutting season has passed the peak.

The yearlings and fawns keep fat most of the year, except the precocious ones who take part in the rut. The antlers are shed later than those of the does in the spring.

Steers.—Reindeer make good steers as a rule, up to three or four years; after that, if they are worked as sled deer, they lose their smooth appearance, get high in the withers, and become slab-sided. Steers are not nearly as fat as the bucks in July and August; they reach their peak toward the end of September and the beginning of October. The antlers are shed in the spring about the same time as the does. The velvet is not peeled off the antlers. They are large and flat and the tines are blunt and round-ended.

Deposition of Fat.—Reindeer fat in a growing condition has a pinkish tinge due to vascularity. When fully formed, it is hard and pure white. Reindeer milk and cream are chalky white. The back fat fills in a space from the region over the kidneys, where it is thin, to the root of the tail, like two large wedges, as there is a furrow along the back over the spine. The

—*Photo by S. Hadwen*

Fig. 1—Reindeer fawn. Ordinary growth of antlers. Compare with figure 2.

greatest thickness of fat is 2½ to 3 inches.

Caul Fat.—This occurs in small amount and can usually be held in both hands.

Kidney Fat.—The amount is less than that of a fat sheep.

Rib Fat.—There is a fair amount on the inside of the sternum and between the ribs, up as far as the cartilaginous nodes.

External Fat.—On the hams and shoulders, there is little fat except for the back fat on the hams. The shoulders are lean. Between the muscles, there is very little internal fat.

Weight of Carcasses.—In 1935, when the deer had just been delivered, the average dressed weight for steers was 150 lb. In 1939, it had risen to an average of 167 lb., some carcasses weighing 200 lb.

In the Anderson River herd derived from the main herd in 1938, seven 2- to 3-year-old steers averaged 190 lb. In the same herd 10 fawns 3 months and 1 week old averaged 54 lb. dressed.

Reindeer Ranges.—In Canada, the ranges are new in a territory where the caribou were killed off a long time ago. Thus, the grazing is at its best and it is not supplemented in any way, either by drying or preserving the natural forage, or by the importation of food of any kind. The weights given above, therefore, were not influenced by artificial feeding.

RATE OF INCREASE

These figures do not include the animals which died from various causes or were killed for food (bucks and steers), as I do not have accurate figures. I have one note relating to 25 fawns which died young and 15 others which were killed for their skins. My figures, therefore, are conservative:

The rate of increase is high for reindeer and means that the herd will double itself in three years.

EARLY FERTILITY

In 1939, when I visited our Canadian reindeer herds in the Western Arctic, I was asked to determine, if possible, the reason that an increasing number of late fawns were being born each year. It was ascertained that this was mainly due to yearling does having fawns. In most reindeer herds, it is rare that the does have fawns before they are 2 years old. It was difficult to get accurate figures or percentages at first, but in 1939 about 40 yearling does had fawns in one of the herds.

Owing to the remoteness of our reindeer herds, it has been difficult for me to secure all of the facts relating to the young does. Mr. Stanley Mason, who has charge of both reindeer herds, had to go from one

ANDERSON RIVER COUNT OF HERD 1

Adult does	692	*Increase in Size of Herd*	
Adult bucks	68	Original herd 1938	950
Adult steers	338	1939	1196 ....23%
Yearling does	109	1940	1559 ....31%
Yearling bucks	100	1941	2093 ....34%
Fawns, female	264 } 595		
Fawns, male	291 }	25 died young	
Fawns, counted outside corral	40	15 killed for skins	
Mixed deer counted outside corral	153	—	
Old does to be slaughtered	38	635	
	2093	Birth Rate	91 %

HERD 1, ANDERSON RIVER

Yearling Does Having Fawns (1941)

Born	April 27	1
	April 29	2
	May 1	1
	May 9	1
	May 14	1
	May 20	1
	May 23	1

At the annual roundup, July 28-29, twenty-two were counted, showing that some of the fawns were born at dates later than those recorded above.

herd to the other and was unable to secure the late dates of birth in herd 1. I should repeat that the antlers of reindeer are secondary sex organs which reveal many facts to a reindeer man. The fawns have two long tines pointing forward and downward and two long beams curving forward and upward (fig. 1). The young mothers should, therefore, reveal changes in their antlers, such as branching, if they are going to mate, and they should also peel the velvet off in contrast with the nonbreeding fawns which do not. Mr. Mason has given me two photographs showing unmistakable maturity in two fawns, yet retaining some of the characters of fawn antlers. This is to be expected, however, because there could not be functional ovarian development until the doe has grown to a larger size (fig. 2).

Mr. Mason and his Eskimo assistants have noted the following: The young does mate with mature bucks and the young bucks with mature or old females; the latter observation coincides with what I have noted. All of the herders state that the young does make better mothers than the older ones. This whole question is of great interest as regards the health and increase of the herds. It can not be doubted that the early fawning habit is increasing with a general improvement in the herd, which indicates that health, high fertility, and vigor go together. My former partner, Mr. L. Palmer, informs me that, in Alaska, yearling does with fawns have been observed in a proportion as high as 5 per cent. This was years ago, when the herds were small and the ranges new. Annual increases of 33 per cent were reported then, but now the annual increase has dropped to 20 per cent.

It would appear that early fertility and

—*Photo by S. Mason in Western Canadian Arctic.*
Fig. 2—Precocious fawn. Note general similarity to figure 1 with extra branching at top.

the increase in weight of the deer are due to the excellent range, the climate, and possibly other factors. The question arises as to what will be the first signs of deterioration. Will the ranges show a gradual

HERD 2, ANDERSON RIVER

Yearling Does Having Fawns (1941)

Born	May 10	1
	May 20	1
	May 23	1
	May 26	1
	May 27	1
	May 29	1
	June 1	1
	June 3	1
	June 6	1
	June 7	1

The records show that some of the fawns were born later than the proper season, which usually ends about May 15. This indicates that the does mated late, as expected.

elimination of palatable plants or will a reduced fertility in the herd be manifest before any signs of damage to the range or reduction in the average weight of the animals? It would seem likely that lessened fertility will be the first sign.

Studies on the Nature of Anaplasma*

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FOR A NUMBER of years, the nature of the etiologic agent of bovine anaplasmosis has been a subject of considerable interest to research workers. Although most investigators who have studied cases of anaplasmosis believe that the "anaplasm" is a protozoan parasite, the lack of satisfactory demonstration of a developmental cycle is sometimes considered as evidence that the etiologic agent is an unknown organism, or possibly a virus. The importance of knowing the nature of the causative factor is emphasized by the need for a rational approach to the problems of treatment, determination of the natural means of dissemination, and institution of control measures.

In studies conducted since 1939 at the Zoölogical Division's laboratory at the Beltsville Research Center, Beltsville, Md., special attention has been given to the types of bodies occurring on or inside the erythrocytes of cattle experimentally infected with *Anaplasma marginale*. Most of these extra- and intra-erythrocytic structures have been described in isolated observations by previous investigators, but there has been no convincing attempt to correlate these observations. The present study has revealed certain relationships between the bodies occurring outside the red cells and those definitely located within them and has indicated growth and multiplication of the anaplasms. These observations appear to cast additional light on the nature of the etiologic agent, strongly suggesting protozoan affinities for this parasite.

MATERIALS AND METHODS

Sixteen bovine animals of mixed breed, ranging in age from 4 months to 5 years, served as sources of blood for the studies of the anaplasm. Three of them were infected as the result of mechanical transmission by the horsefly, *Tabanus sulcifrons*, while the others were infected by the intravenous injection of 9 to 4,000 cc. of blood from an infected animal. In several infected animals, blood smears were made daily from the time of inoculation for periods of one to four months. In the remainder, blood smears usually were made two or three times per week until anaplasms were found, and daily thereafter until the anaplasms became few in number after the crisis had passed, or until the host animal died or was slaughtered for postmortem examination.

Blood smears were made from fresh blood, oxalated, or citrated blood which had stood at room temperature for periods of 1 to 30 days, blood treated with various hemolyzing agents, and blood previously frozen. Some smears were dried in the air immediately after they were made, and others were treated with the fumes of glacial acetic acid, or formalin, previous to drying. Wright's, Giemsa's, May-Grünwald's, Heidenhain's iron hematoxylin, and permanganate blue stains were used. Wright's stain followed by Giesma's stain was used extensively in routine work; each blood smear was stained for 3 minutes in Wright's stain and, after a rinse in tap water, was placed in dilute Giemsa's stain for from 5 to 15 minutes.

The hemolyzing agents included saponin, sodium taurocholate, hydrochloric acid, acetic acid, and citric acid, all of which were made up in sufficient concentration in physiologic saline to produce hemolysis in 1 to 30 minutes when one part of fresh, oxalated, or citrated blood was added to two parts of the hemolyzing solution. The effects of the hemolyzing solution consisting of osmic and acetic acid in distilled water, as mentioned by Gomes de Faría,[1] also were investigated.

The agent used most extensively in this study was a 0.5 per cent solution of acetic acid in physiologic saline, which caused the anaplasms to swell and some to rupture before the erythrocytes were completely hemolyzed. The technic consisted of placing 1 cc. of blood, with or without an anticoagulant, in a test tube containing 2 cc. of the acetic acid-hemolyzing solution. The contents of the tube were then thoroughly mixed by shaking, and at intervals of about one

*From the Zoölogical Division, Bureau of Animal Industry, U. S. Department of Agriculture.

†Transferred to U. S. Public Health Service, Baltimore, Md., December 1940.

[1]Gomes de Faría, José: Estudios sobre la "tristeza" de los bovinos. Piroplasmosis y anaplasmosis. Rev. Inst. Bact. Dept. Nac. Hig., Buenos Aires, v (1928), pp. 429-469.

minute over a period of ten minutes or longer, small drops of the mixture were withdrawn for smears. To prevent crenation and vacuolation of the erythrocytes, the smears were quickly dried by an electric fan. The usual method of staining was practiced, with the exception that the distilled water for diluting the stains was buffered to about pH 8.2 by dibasic sodium phosphate (Na_2HPO_4).

Measurements were made with a camera lucida and a scale made up of light bars of different lengths, according to the method previously described by the authors.[2] In order to avoid duplication in measuring and to have a more representative sample of infected erythrocytes, two glass fibers were arranged parallel to each other in the ocular of the microscope and as the blood smear was moved across the stage of the microscope, the images of all erythrocytes between the glass fibers were carefully examined and classified. This method was used in sampling blood smears showing 0.25 or more per cent of the total number of erythrocytes to be infected. When the infection was lighter and it was necessary to examine an entire slide to find an appreciable number of anaplasms for comparative studies, all erythrocytes in the entire field were examined as the slide was moved. Each anaplasm was carefully measured and the relative abundance of anaplasms of different sizes was determined from these tabulations. By the latter method, it was not possible to estimate accurately the total number of erythrocytes infected. Hematocrit readings were made according to the method of Wintrobe.[3]

OBSERVATIONS

In all of the cases studied, two types of anaplasms were recognized in ordinary blood smears made at the time the blood was let and stained with the combination of Wright's and Giesma's stains. The two types were (1) those which were spherical in outline with smooth contours, measuring 0.2 to about 0.5μ in diameter, and designated in this paper as smooth anaplasms, and (2) those which were roughly spherical or ovoidal, occasionally cuboidal, measuring about 0.6 to 0.9μ in diameter, and designated as rough anaplasms.

Careful examination of these two types with ordinary and dark-field illumination, especially in blood smears 5 to 12 months old in which the stains were faded, gave the impression that the smooth anaplasms were each made up of a single mass of homogenous material, while a number of small, spherical bodies of equal size, designated as sporoid bodies, were observed in the rough anaplasms. The arrangement of the sporoid bodies was difficult to determine in most anaplasms, but in some cases, especially in smears made from the peripheral blood and material from the spleen of severe cases, a large number of the rough anaplasms appeared to be slightly swollen and the sporoid bodies they comprised were more widely separated than in this type of anaplasm encountered in the majority of cases of anaplasmosis. In a few instances, rough anaplasms were observed in which four sporoid bodies were arranged in a tetrad around a clear, central space on the upper surface of the anaplasm, and a second tetrad of sporoid bodies appeared to be present almost directly underneath the first (fig. 1). In rare instances, sporoid bodies were arranged in a rough circle around a large, clear, central space, anaplasms in which this arrangement was present measuring 0.8 to 0.9μ in diameter.

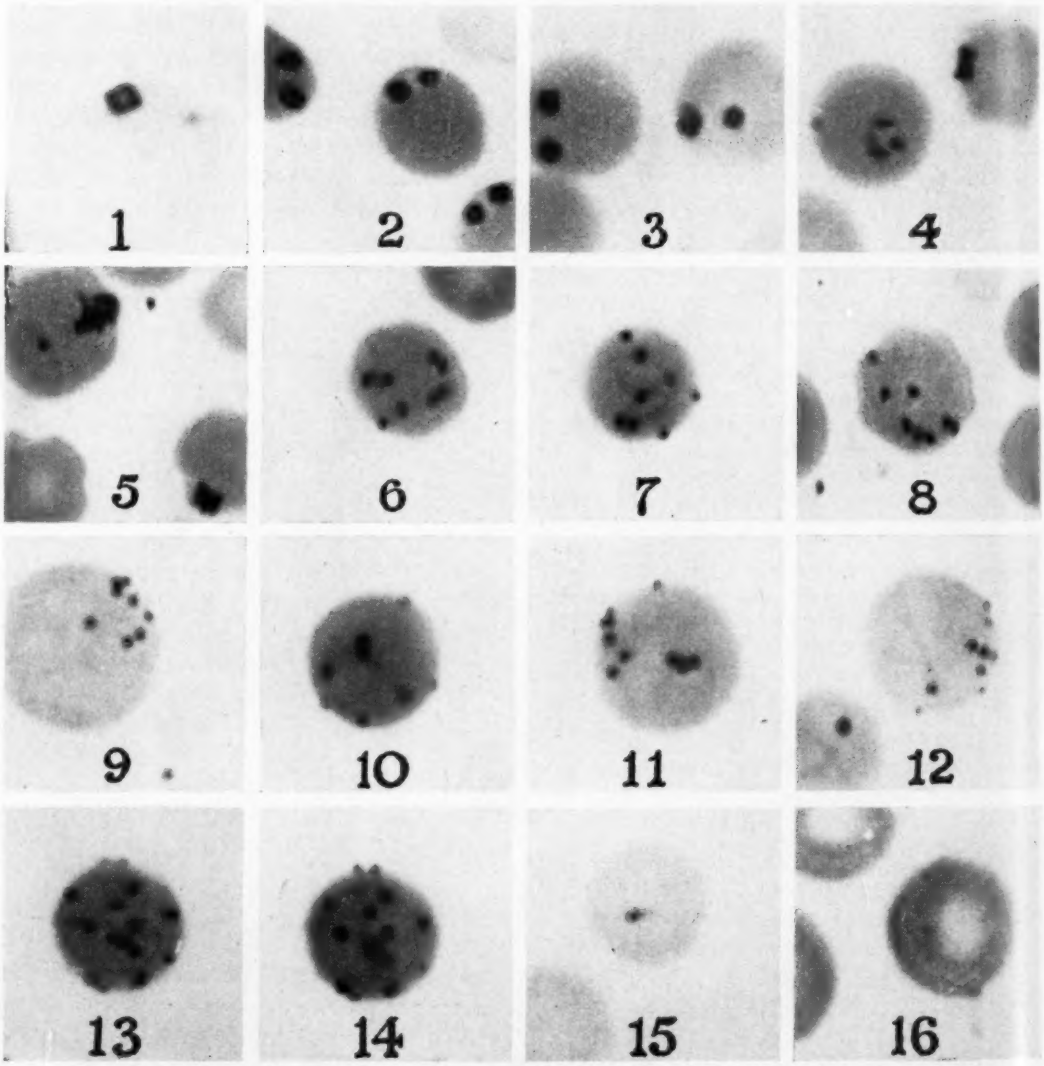
In a few erythrocytes from acute cases, especially when a large number of the erythrocytes contained rough anaplasms and many of the infected erythrocytes contained three or more anaplasms of equal or unequal size, a large number of small, spherical bodies similar in size and shape to the sporoid bodies were found scattered throughout the stroma of the red cells. These bodies were similar to the small, smooth anaplasms, measuring about 0.2μ in diameter. Since the vast majority of both the smooth and rough anaplasms ordinarily occurred in a more or less marginal position in the infected erythrocytes, and because of the relatively large number of these small bodies scattered throughout the stroma of the erythrocytes, it seems probable that these small bodies resulted from the rupturing of one or more rough anaplasms and, therefore, represented sporoid bodies rather than smooth anaplasms.

Besides the two types of anaplasms, the

[2] Lotze, J. C., and Yiengst, M. J.: An apparatus for measuring microscopic objects. Sci., xciii (1941), pp. 45-46.

[3] Wintrobe, M. M.: A simple and accurate hematocrit. J. Lab. & Clin. Med., xv (1929), p. 287.

small spherical bodies scattered in the stroma of the erythrocytes and similar in appearance to the sporoid bodies, the nuclei of normoblasts, Jolly bodies, and punctate basophilic stippling, other intra-erythrocytic bodies found consisted of "Bartonella-like" structures which were rare and occurred in greatest numbers during the "incubation" period of a few cases. These structures do not appear to be directly involved in the present study and will be included in a later report. The Jolly bodies and basophilic stippling were distinguishable from the anaplasms on the basis of appearance and staining reactions. It is interesting to note that the Jolly bodies were found, associated with other symptoms of anemia, only close to the crisis of the disease and that anaplasms were not detected in erythrocytes containing such



Photomicrographs of Structures Found on and Inside of Bovine Erythrocytes During Anaplasmosis.

Fig. 1—Anaplasm showing 4 "sporoid" bodies (ordinary blood smear). Figs 2 to 14—Anaplasms and constituent parts after acetic acid treatment (figs. 13 and 14) same erythrocyte photographed at different levels). Fig. 15—Extra-erythrocytic body on upper surface of erythrocyte. Fig. 16—Extra-erythrocytic body on outer margin of erythrocyte.

bodies or in other immature erythrocytes which showed basophilic stippling or polychromasia.

Observations on smears made of blood treated for one to five minutes or longer with 0.5 per cent acetic acid solution to produce swelling of anaplasms and separations of their component parts without destroying the parasitized cells, showed the following:

1) No abnormal structures appeared in the erythrocytes of either uninfected or "carrier" bovine animals which were free of anaplasms.

2) When hemolysis was controlled so that apparently no erythrocytes were destroyed, the number of whole or broken anaplasms was the same as in untreated blood.

3) The spherical anaplasms with smooth contour showed no evidence of internal structural organization; each appeared to be a single mass of homogenous material.

4) The anaplasms with irregular contour were affected by this treatment in various ways (fig. 2-14). Sometimes, the small, sporoid bodies comprising the anaplasm were arranged in a variously shaped, flattened, compact mass; in other cases, these bodies were more widely separated and arranged in a circle, and in many cases, the sporoid bodies were so widely separated in the stroma of the erythrocyte that the identity of the anaplasm was no longer apparent (fig. 6-14).

5) In blood containing before treatment not more than one anaplasm per infected erythrocyte, there were significantly fewer anaplasms showing irregular contour following treatment. Instead of the expected number of anaplasms in this treated blood, a certain proportion of the erythrocytes regularly contained groups of the small, spherical bodies similar to sporoid bodies. These bodies appeared regularly in a group of eight in each erythrocyte when sufficiently separated from each other to be counted with certainty.

6) In blood containing before treatment more than one anaplasm per infected erythrocyte, various combinations of structures were found following treatment. For example, two or three spherical anaplasms with smooth contour were found in an erythrocyte which also contained eight widely separated bodies similar to the sporoid bodies. Occasionally, nine or ten bodies similar to the sporoid bodies were found in a single erythrocyte. The examination of slides made from the untreated portion of this blood sample showed that two or more small, smooth anaplasms occasionally occurred in an erythrocyte which contained one of the rough ana-

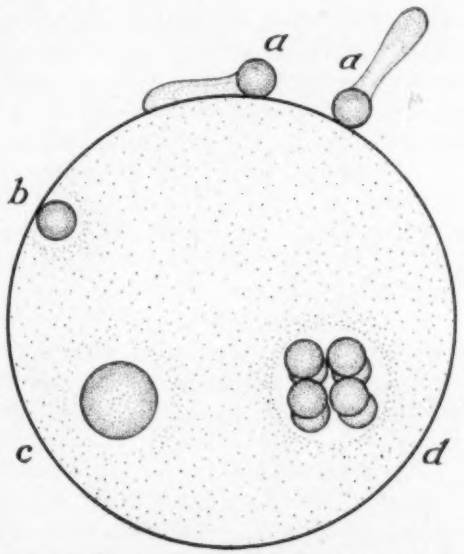


Fig. 17—Schematic representation of the types of anaplasms and their possible origin; *a*. extra-erythrocytic form; *b*. young or small anaplasm; *c*. older or intermediate size anaplasm; *d*. "mature" anaplasm showing 8 "sporoid" bodies.

plasms. In blood containing before treatment three or four anaplasms, mostly of the rough type, sometimes 16 or 24 bodies similar to the sporoid bodies were observed in a single erythrocyte following treatment (figs. 13, 14).

From these observations, it was indicated that each of the large or rough anaplasms was composed of eight small spherical or sporoid bodies and that the smaller or smooth anaplasms were single units of material. Results similar to these also were obtained when citrated or defibrinated blood containing anaplasms was allowed to

stand at room temperature, at 37 C., and also when frozen immediately after letting. Although fewer anaplasms appeared to be affected, some were observed to be swollen, while in some erythrocytes, the sporoid bodies were more widely separated and occurred in various configurations.

Finding the constituent parts or sporoid bodies of an anaplasm widely scattered throughout the stroma of an erythrocyte gave the impression that the bodies were hurled by force from the anaplasm. However, a cell or limiting membrane around the large, irregular-shaped anaplasms was not demonstrable. A possible explanation for the scattering of these bodies was found when the swelling action of the erythrocytes treated with acetic acid was studied with dark-field illumination. Instead of a gradual change in the size of the erythrocytes, a series of short, violent expansions took place.

At the onset of and at all times during the phase of the disease when anaplasms were present, designated in this paper as the anaplasm phase, a few small, frail, rod-shaped structures were found attached to mature erythrocytes in the blood stream (fig. 15, 16, 17a). Each structure measured about 1μ in length and, except for a knob-like body at one end, measured about 0.1 to 0.2μ in diameter. The terminal knob-like structure, which measured about 0.2 to 0.3μ in diameter, stained dark pink with a purplish tint with Wright's and Giesma's stains; the remainder of the rod-shaped structure stained a light pink. A vacuolated or clear space occurred in almost a central position.

When viewed with ordinary illumination, the dense, spherical, knob-like portion of this extra-erythrocytic body was usually the first to attract the observer's attention. Usually, the remainder of the structure or tail-like portion was not readily observed until adjustments were made in the illumination. By using different color filters, it was found that the detailed structure of this form could be more readily discerned when the blue-glass was removed from the microscope lamp and a bottle containing a lemon-colored fluid was placed between the lamp and the microscope.

A careful study of this extra-erythrocytic form always showed it to be either free in the blood plasma or attached to the surface of erythrocytes. Usually, it was found attached at the outer margin of the erythrocyte (fig. 16), but in a few cases, it occurred on the more central portion of the upper surface (fig. 15). When anaplasms were present inside the erythrocyte and one of these extra-erythrocytic forms was on the upper surface of the same red blood cell, often the first imression to the observer was that both types of structures were within the erythrocyte. This impression was often due to the depression on the upper surface of the erythrocyte which placed both the anaplasm and the extra-erythrocytic form on the same focal plane of the microscope.

In examining blood smears made daily during the "incubation" period and the phase of the disease characterized by the presence of anaplasms, certain phenomena common to all cases were noted. In addition to the extra-erythrocytic bodies, the number of which forms found attached to the erythrocytes was correlated with the number of small anaplasms appearing in the erythrocytes at a particular period, measurements of the anaplasms and the tabulation of infected erythrocytes on the basis of these measurements showed that the anaplasms apparently changed in size. This was especially noticeable at the onset of the anaplasm phase in some of the acute cases and in a few instances at various periods in the more "chronic" or less severe types. For example, on a particular day the majority of infected erythrocytes from a bovine animal contained small anaplasms measuring 0.2μ in diameter, and on the following day anaplasms measuring 0.3 and 0.4μ in diameter. On the third day, the majority of the infected erythrocytes contained anaplasms measuring 0.4 and 0.5μ, and on the fourth day there was an increase in the number of erythrocytes containing rough anaplasms which measured 0.6 to 0.7μ. On the fifth day, only a relatively few or no rough

anaplasms were present, while the majority of infected erythrocytes contained smooth anaplasms of a smaller size.

In the majority of cases, a progressive change in the size of the anaplasms was not as apparent as in the instances mentioned above, and anaplasms of various sizes seemed to be present in almost equal proportions. However, the daily tabulation on the basis of size showed that in these cases, also, there were "size rhythms" which were especially apparent at certain times. These findings are illustrated in table I, which contains certain excerpts from a case-history study of anaplasmosis in a splenectomized susceptible calf which was inoculated at the age of about 5 months and in which the disease developed rapidly.

TABLE I—Data on Anaplasmosis in a Splenectomized Susceptible Bovine Animal (No. 197, Inoculated Intravenously on April 10, 1940, with 90 cc. of Defibrinated Blood from "Carrier" Animal 194)

Date (1940)	Temp. (F.)	Hematocrit Reading (Volume Per Cent)	Anaplasms per Infected Cell (Numerical Average)	Erythrocytes Infected (Per Cent of Total)	Grouping of Erythrocytes According to the Largest Anaplasm Found in Each Red Cell (Per Cent of Total Erythrocytes)						Number of Red Cells Examined
					0.2 μ	0.3 μ	0.4 μ	0.5 μ	0.6 μ	0.7 μ	
Apr. 18	102.4	32.5	1.0	+	+*	+					(Entire slide)
19	101.9	31.5	1.0	+	+*	+	+				(Entire slide)
20	102.2	32.5	1.0	+	+*	+	+	+			(Entire slide)
21	102.0	35.0	1.0	+	+*	+	+	+			(Entire slide)
22	101.8	33.0	1.0	+	+	+*	+	+	+		(Entire slide)
23	102.8	32.5	1.0	+	+	+*	+	+			(Entire slide)
24	101.8	33.0	1.0	+	+	+	+	+	+*		(Entire slide)
25	101.6	34.5	1.0	+	+	+*	+	+	+		(Entire slide)
26	101.8	34.5	1.+	.25			.2*	.05			2,000
27	101.8	34.0	1.+	.864	.066	.133	.133	.4*	.066	.066	1,500
28	101.2	32.5	1.+	2.1	.4	.3	.2	.1	1.1*		1,000
29	102.4	36.0	1.+	6.6	1.6	1.0	2.0*	1.6	.4		1,000
30	102.6	33.5	1.05	10.5	1.6	1.0	.4	1.0	6.5*		500
May 1	101.9	31.5	1.20	19.0	1.0	3.4	9.2*	4.0	1.4		500
2	104.8	30.0	1.33	37.2	10.0	4.2	2.6	7.0	13.4*		500
3	103.2	24.0	1.84	55.6	10.4	26.0*	15.0	3.8	.4		500
4	105.4	19.5	1.83	69.4	8.0	10.0	44.0*	6.8	.6		500
5	104.2	9.5	1.60	61.2	4.2	14.0	13.6	25.0*	4.4		500

*Plurality group.
+ = A number smaller than 0.05.

The calf was born Nov. 15, 1939, and splenectomy was performed March 25, 1940. On Apr. 8, 1940, the calf was inoculated intravenously with 90 cc. of defibrinated blood from "carrier" 194. The first anaplasms were found on April 18, or ten days after inoculation. Although there was a fairly rapid increase in the number of anaplasms after this time, only 0.25 per cent of the total number of erythrocytes in the host's blood stream were infected April 26, or eight days later. From April 18 to April 25, or during the first eight days that anaplasms were found, there was only one per infected erythrocyte. From April 26 to May 3, inclusive, there was a gradual increase in the numerical average of anaplasms per infected erythrocyte; the greatest increase came between April 30 and May 3 and was correlated with a marked increase in the percentage of erythrocytes infected. It will be noted that the size of the anaplasms occurring from day to day varied and that these variations tended to show definite "size rhythms." These "size rhythms" established at the onset of the anaplasm phase of the disease, when only one anaplasm occurred in an infected erythrocyte, were still apparent near the crisis of the infection, as shown in table I, when the numerical average of the number of anaplasms per infected erythrocyte rose above 1 to 1.84 on May 3, and where only the largest anaplasm in each infected erythrocyte was considered.

It is interesting to note (table I) that the infection in this case was well advanced before the hematocrit readings indicated a loss in the total volume of erythrocytes in the host's blood stream and that fever was first manifested May 2, or on the four-

teenth day after anaplasms were first found.

The extra-erythrocytic forms were first detected in the blood of this animal on April 17, or on the day previous to the first detection of anaplasms. These forms were found throughout the anaplasm phase and were especially noticeable at the time of the rapid increase in the number of anaplasms. Although in most instances the bodies occurred on the periphery of erythrocytes which contained no anaplasms, they occurred also on the periphery of erythrocytes which contained one or more anaplasms. Occasionally, two of these bodies were observed attached to the same erythrocyte.

DISCUSSION

Theiler's[4] description of the anaplasm as a parasite composed of chromatin or nuclear material undoubtedly stimulated investigators to interpret the anaplasm as nuclear remnants of the erythrocyte or other products formed as the result of the presence of some unknown agent, such as a virus. Schilling and Meyer[5] presented certain criticisms of the theory that the etiologic agent of anaplasmosis is a protozoan parasite, one criticism being that a developmental cycle has not been satisfactorily demonstrated. From correlation of observations reported in this paper with isolated descriptions of certain structures reported in the literature, it seems apparent that there is a developmental cycle for the anaplasm.

Although Smith and Kilborne[6] included the anaplasm as a stage in the life history of the etiologic agent of Texas fever, their description of the anaplasm is of interest:

> In the fresh preparations of blood this small stage of the parasite is as a rule invisible. Rarely we may observe it on the very border of the corpuscle as a round pale spot about 0.5μ in diameter, which does not change its place. When dried films of blood are stained in alkaline methylene blue the parasites appear as round coccus-like bodies from 0.2 to 0.5μ in diameter and situated within the corpuscle on its border. They sometimes appear as if situated on the border but outside of the corpuscle. As a rule, only one is found in a corpuscle. In many cases, a division of the coccus-like body into two could be clearly made out. The separation was noticeable as a paler line and a constriction at either end similar to the division of certain micrococci. This division usually appeared in all bodies of a preparation from one case, but could not be noticed in any preparation of perhaps the next case.
>
> These bodies stain as well as the larger pyriform bodies in basic aniline dyes and in haematoxylin. They do not stain in acid dyes such as eosin or in Ehrlich's dye for neutrophile granules. When this stain is employed the corpuscles, beautifully tinted, show a small round unstained spot where the parasite is situated. When the dried films are treated with dilute acetic acid the corpuscles fade out, while these coccus-like bodies remain behind and stand out prominently.

Since Theiler[4] described the anaplasm, a number of investigators have reported the occurrence of various types of bodies within and outside the erythrocytes in the course of the disease. For example, Sieber[7] described the anaplasms as "parasites" which appear as "purple-bluish granules of different sizes," which show "(after lengthy staining) a small black body in the center or on the margin of the granule" and in "double forms, a line of connection between the two central bodies is distinctly visible, showing dumb-bell formations." De Kock and Quinlan[8] mentioned anaplasms having contours which here and there showed slight protrusions, as if sprouting, and Boynton[9] mentioned anaplasms as having "protuberances which appear to be buds." Quevedo,[10] Descazeaux,[11] Gomes de Faría[1] and Dikmans[12] mentioned the presence of numerous

[4]Theiler, A.: *Anaplasma marginale* (gen. and spec. nov.). The marginal points in the blood of cattle suffering from a specific disease. Rpt. Govt. Vet. Bact., Transvaal Dept. Agr., 1908-09 (1910).

[5]Schilling, C., and Meyer, K. F.: Piroplasmosen. Handb. Path. Mikroörganism (Kolle u. Wassermann), 3. Aufl., Lief. 9, viii (1927), pp. 1-94.

[6]Smith, T., and Kilborne, F. L.: Investigations into the nature, causation, and prevention of Texas or southern cattle fever. BAI, USDA Bul. 1 (1893), pp. 1-301.

[7]Sieber, H.: *Anaplasma marginale* (Theiler). Rpt. Govt. Vet. Bact., Union of South Africa, 1909-10 (1911), pp. 104-116.

[8]de Kock, G., and Quinlan, J.: The difference between Anaplasma and Jolly bodies. S. African J. Sci., xxiii (1926), pp. 755-759.

[9]Boynton, W. H.: Further observations on anaplasmosis. Cornell Vet., xxii (1932), pp. 10-28.

[10]Quevedo, J. M.: Notas sobre la tristeza. Rev. Centro Estud. Agronom. y Vet., Univ. Buenos Aires, vii (1914), pp. 10-16.

small bodies arranged in various configurations in the erythrocytes.

In the light of the present investigations, from a morphologic standpoint two types of anaplasms are present, namely, smaller anaplasms measuring about 0.2 to 0.5μ and composed of a single mass of substance, and anaplasms measuring about 0.6 to 0.7μ or greater, each composed of eight small, spherical, sporoid bodies arranged in a compact mass. The anaplasms believed by Smith and Kilborne to represent incomplete division, those mentioned by Sieber as containing a "central body," and those mentioned in reference to De Kock and Quinlan, and Boynton, are apparently anaplasms of the larger, or rough, type.

In the present studies on blood treated with the acetic acid hemolyzing solution, some of the large, rough anaplasms appeared slightly swollen, in which cases the eight small, sporoid bodies contained in each were more readily discerned with dark-field illumination, while other anaplasms were apparently ruptured by the treatment and the sporoid bodies appeared in various configurations not unlike those mentioned by Quevedo, Descazeaux, Gomes de Faría, and Dikmans. It is interesting to note that figures 67 and 68 in the report of Gomes de Faría are photomicrographs of blood from "cultures," in which a number of small, spherical bodies are very similar in structure and arrangement to those found in some of the photomicrographs included in this paper.

Although not definitely stated, growth of the anaplasm was apparently postulated by Smith and Kilborne[6] to account for the change in size of the anaplasm from that of the "(hypothetical) swarm-spore." In an account of the progress of the disease in a calf, Sieber[7] stated that "gradually small parasites developed into larger bodies" and that "the blood corpuscles showed sometimes double or triple infections," but other than the statement that "the erythrocytes, invaded by anaplasms, are kept back by the spleen, where they will eventually be dissolved," his views as to the presence of a developmental cycle are not definite.

From mention in the literature of "small points" or marginal bodies occurring at certain times, especially at the onset of the anaplasm phase, and the occurrence of larger anaplasms afterward—a phenomenon also found in the present investigations—it seems probable that growth of the anaplasm does take place. From the tabulation of the size of anaplasms occurring in the erythrocytes from day to day, it appears that the smaller anaplasms undergo growth which is followed by multiple division or segmentation resulting in a rough anaplasm which is more or less irregular in outline and which contains a definite number of small, sporoid bodies. The data in table I indicate that an infected erythrocyte disappears from the blood stream soon after the multiple division occurs in any one of the anaplasms which may be contained in the erythrocyte, and that the time necessary for the growth and division is probably three to four days.

To account for the presence of the anaplasm in an erythrocyte, Smith and Kilborne[6] postulated that a "(hypothetical) swarm-spore" entered the erythrocyte. Sieber[7] apparently believed that the small anaplasms entered the erythrocytes. Helm[13] reported some interesting experiments on culture of anaplasms, in which it seemed probable that the anaplasm arose from a form outside the erythrocyte. One of these structures, the "Birnenform," appears to be identical with the extra-erythrocytic form observed in the present investigations, the structure "que presentaban un aspecto de virgula" described by Gomes de Faría,[1] and with a similar structure mentioned by Quevedo,[10] Descazeaux,[11] and the reddish-stained rod forms mentioned by Dikmans.[12] A structure described by Boynton[9] as composed of a portion of the stroma of the erythrocyte, forming a comet-like

[11]Descazeaux, J.: L'anaplasmose au Chili. Bul. Soc. Path. Exot., Paris, xvii (1924), pp. 639-642.

[12]Dikmans, G.: Anaplasmosis. VI. The morphology of Anaplasma. J.A.V.M.A., lxxxiii (1933), pp. 203-213.

[13]Helm, R.: Beitrag zum Anaplasmen-Problem. Zeit. f. Infekt. d. Haust., xxv (1924), pp. 199-226.

tail on the side of a marginal body, also may be of the same nature.

Possibly, the extra-erythrocytic form observed in this study may be the forerunner of the small anaplasm, since it always occurred on the periphery of a certain number of mature erythrocytes and not on normoblasts or other immature erythrocytes showing basophilic stippling and polychromasia. Other structures observed frequently in blood from cases of bovine anaplasmosis, and mentioned in a recent report (Lotze and Yiengst[14]) under the name of eperythrozoa, resemble in certain respects this extra-erythrocytic form.

SUMMARY AND CONCLUSIONS

The order and regularity of the occurrence of the different-sized and morphologic types of anaplasms in the course of anaplasmosis suggest that the anaplasm is a parasite which invades the erythrocyte, undergoes a period of growth, and eventually undergoes multiple division resulting in the production of eight small, spherical bodies in each anaplasm. The rapid disappearance of the large, irregular-shaped anaplasms from the blood stream of the host animal suggests that these anaplasms are in some way responsible for the destruction of the erythrocytes containing them.

Evidence from tabulations of infected erythrocytes based on the size of the anaplasms indicates that an infected erythrocyte does not remain in the blood stream of the host longer than three or four days.

The occurrence of a certain extra-erythrocytic body during the anaplasm phase of the disease and the attachment of this structure to mature erythrocytes only, usually in a marginal position on the erythrocytes, suggests that this form constitutes the stage of the parasite which enters the erythrocyte and develops into the anaplasm.

[14]Lotze, J. C., and Yiengst, M. J.: "Eperythrozoönosis" in cattle in the United States. No. Am. Vet., xxii (1941), pp. 345-346.